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|      | SPLEEN |  |  | KIDNEY |  |  | HEART |  |  | BRAIN |  |  |
|------|--------|--|--|--------|--|--|-------|--|--|-------|--|--|
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| M3 — |        |  |  |        |  |  | —     |  |  |       |  |  |
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(57) Abstract: The present invention provides means and strategies for treating the lysosomal storage disorder alpha-mannosidosis by enzyme replacement therapy. In particular, the invention pertains to the reduction of stored neutral mannose-rich oligosaccharides in cells within the central nervous system. Accordingly, the present invention provides the use of a lysosomal alpha-mannosidase for the preparation of a medicament for reducing the intracellular levels of neutral mannose-rich oligosaccharides in cells within one or more regions of the central nervous system. The invention further provides a formulation of lysosomal alpha-mannosidase for use as a medicament for reducing the intracellular levels of neutral mannose-rich oligosaccharides in cells within one or more regions of the central nervous system. Also, the invention provides a method of reducing the intracellular levels of neutral mannose-rich oligosaccharides in cells within one or more regions of the central nervous system of a subject, said method comprising administering to said subject a preparation of lysosomal alpha-mannosidase. Finally, the invention pertains to methods of producing, isolating, and purifying an alpha-mannosidase as well as the use of an alpha-mannosidase, which is obtainable according to these methods.

WO 2005/094874 A1

**WO 2005/094874 A1**

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WO 2005/094874

PCT/DK2005/000228

## USE OF ALPHA-MANNOSIDOSE FOR THE CORRECTION OF ABNORMAL LYSOSOMAL STORAGE IN THE CNS

### 5 FIELD OF INVENTION

The present invention provides means and strategies for treating the lysosomal storage disorder alpha-mannosidosis by enzyme replacement therapy. In particular, the invention pertains to the reduction of stored neutral mannose-rich oligosaccharides in cells within the central nervous system.

10

### BACKGROUND OF THE INVENTION

#### Alpha-mannosidosis

- Alpha-mannosidosis is a recessive, autosomal disease that occurs world wide with a frequency of between 1/1.000.000 and 1/500.000. Mannosidosis is found in all ethnic groups in Europe, America, Africa and also Asia. It is detected in all countries with a good diagnostic service for lysosomal storage disorders, at a similar frequency. They are born apparently healthy, however the symptoms of the diseases are progressive. Alpha-mannosidosis displays clinical heterogeneity, ranging from very serious to very mild forms.
- Typical clinical symptoms are: mental retardation, skeletal changes, impaired immune system resulting in recurrent infections, hearing impairment and often the disease is associated with a typical facial characteristics such as a coarse face, a prominent forehead, a flattened nasal bridge, a small nose, and a broad mouth. In the most severe cases (mannosidosis type I) the children suffer from hepatosplenomegaly, and they die during the first years of life. Possibly this early death is caused by severe infections due to the immunodeficiency caused by the disease. In milder cases (mannosidosis type 2) the patients usually reach adult age. The skeletal weaknesses of the patients result in the needs of wheeling chairs at age 20 to 40. The disease causes a diffuse dysfunction of the brain often resulting in weak mental performances that excludes anything but the most basic skills of simple reading and writing. These problems associated with hearing inabilities and other clinical manifestations preclude the patient from an independent life, the consequence being that life long caretaking is needed.

#### Lysosomal alpha-mannosidase

- Alpha-mannosidosis results from a deficient activity of lysosomal alpha-mannosidase (LAMN, EC3.2.1.24). The disease is characterised by massive intracellular accumulation of mannose-rich oligosaccharides, that is oligosaccharides carrying  $\alpha$ 1,2-,  $\alpha$ 1,3- and  $\alpha$ 1,6-mannosyl residues at their non-reducing termini. These oligosaccharides mainly originate

WO 2005/094874

PCT/DK2005/000228

2

from the intralysosomal degradation of glycoproteins with N-linked oligosaccharides. However, some originate from the catabolism of dolichol-linked oligosaccharides and from misfolded glycoproteins redirected to the cytosol for degradation by the proteasome (4, 5). The lysosomal storage is observed in a wide range of cell types and tissues, including  
5 neurons in all brain regions. LAMAN is an exoglycosidase which hydrolyses these terminal, non-reducing alpha-D-mannose residues in alpha-D-mannosides from the non-reducing end during the ordered degradation of the N-linked glycoproteins (Aronson and Kuranda FASEB J 3:2615-2622. 1989). The human enzyme is synthesised as a single polypeptide of 1011 amino acids with a putative signal peptide of 49 residues that is processed into  
10 three main glycopeptides of 15, 42, and 70 kD. (Nilssen et al. Hum.Mol.Genet. 6, 717-726. 1997).

The lysosomal alpha-mannosidase gene

The gene coding for LAMAN (MANB) is located at chromosome 19 (19cen-q12), (Kaneda et  
15 al. Chromosoma 95:8-12. 1987). MANB consists of 24 exons, spanning 21.5 kb (GenBank accession numbers U60885-U60899; Riise et al. Genomics 42:200-207 . 1997). The LAMAN transcript is » 3,500 nucleotides (nts) and contains an open reading frame encoding 1,011 amino acids (GenBank U60266.1).

20 The cloning and sequencing of the human cDNA encoding LAMAN has been published in three papers (Nilssen et al. Hum.Mol.Genet. 6, 717-726. 1997; Liao et al. J.Biol.Chem. 271, 28348-28358. 1996; Nebes et al. Biochem.Biophys.Res.Comm. 200, 239-245. 1994). Curiously, the three sequences are not identical. When compared to the sequence of Nilssen et al (accession # U60266.1) a TA to AT change at positions 1670 and 1671  
25 resulting in a valine to aspartic acid substitution was found by Liao et al. and Nebes et al.

#### Mutations

In 1997 Nilssen et al. (Hum Mol Genet 6:717-726) reported the first disease causing mutation in the fibroblasts from two siblings with mannosidosis in a highly inbred  
30 Palestinian family. In these two patients, the cause of the disease is a point mutation at the gene, with the nucleotide Adenine substituted with Thymidin. Since then, several genetic causes to the disease have been found in man. Today mutations causing alpha-mannosidosis have been characterised in more than 60 patients, mainly in Europeans. These mutations vary both in type, encompassing nucleotide substitutions, deletions and  
35 additions and spanning most of the 24 exons of the gene (Berg et al. Am J Genet 64: 77-88. 1999).

WO 2005/094874

PCT/DK2005/000228

3

The consequences of the mutations on the protein level also vary, from amino acid substitutions that effect both the active site and the folding, to early frame shifts and premature stop codons, resulting in a completely inactive product.

- 5 The amino acid sequence and enzyme structure in a number of different species. When comparing species, many of the human and the bovine mutations found are located to a highly "conserved" region of the mannosidase polypeptide for several species like man, cattle, cat, whale, bird, cod and slime mould, indicating that these regions are very important for the enzyme function.

10

Despite the many different types of mutations, it seems that all the mutations cause the similar complete loss of enzyme activity in cells. Thus, apparently, the variations with respect to clinical severity cannot be explained by variations in the enzyme activity. Rather, these variations are caused by other factors, being attributed to the general ability  
15 of the organism to buffer the obnoxious effects of the accumulating oligomannosides, and by the environments to prevent and to treat infections and other clinical symptoms in the patients.

#### Diagnosis

20

- The diagnosis of alpha-mannosidosis is currently is based on clinical evaluation, detection of mannose-rich oligosaccharides in urine, and direct measurements of alpha-mannosidase activity in various cell types, such as leukocytes, fibroblasts, and amniocytes (Chester et al., In: Durand P, O'Brian J (eds) Genetic errors of glycoprotein metabolism. Edi-Ermes,  
25 Milan, pp 89-120. 1982; Thomas and Beaudet . In: Scriver CR, Beaudet AL, Sly WA, Valle D (eds) The metabolic and molecular bases of inherited disease. Vol 5. McGraw-Hill, New York, pp 2529-2562. 1995).

- Because the symptoms initially often are mild and the biochemical diagnosis is difficult, the  
30 diagnosis is frequently made late in the course of the disease. It is obvious that patients and their families would benefit substantially from an early diagnosis.

#### Animal models

- 35 Alpha mannosidosis has been described in cattle (Hocking et al. Blochem J 128:69-78. 1972), cats (Walkley et al. Proc. Nat. Acad. Sci. 91: 2970-2974, 1994), and guinea pigs (Crawley et al. Pediatr Res 46: 501-509, 1999). A mouse model was recently generated by targeted disruption of the alpha-mannosidase gene (Stinchi et al. Hum Mol Genet 8: 1366-72, 1999).

WO 2005/094874

PCT/DK2005/000228

4

Like in humans alpha mannosidase seems to be caused by specific mutations in the gene coding for lysosomal alpha-mannosidase. Berg et al. (Biochem J. 328:863-870.1997) reported the purification of feline liver lysosomal alpha-mannosidase and determination of its cDNA sequence. The active enzyme consists of 3 polypeptides, with molecular masses of 72, 41, and 12 kD. Similar to the human enzyme it was demonstrated that the feline enzyme is synthesized as a single-chain precursor with a putative signal peptide of 50 amino acids followed by a polypeptide chain of 957 amino acids, which is cleaved into the 3 polypeptides of the mature enzyme. The deduced amino acid sequence was 81.1% and 83.2% identical with the human and bovine sequences, respectively. A 4-bp deletion was identified in an affected Persian cat; the deletion resulted in a frameshift from codon 583 and premature termination at codon 645. No enzyme activity could be detected in the liver of the cat. A domestic long-haired cat expressing a milder phenotype had enzyme activity of 2% of normal; this cat did not possess the 4-bp deletion. Tollersrud et al. (Eur J Biochem 246:410-419 .1997) purified the bovine kidney enzyme to homogeneity and cloned the gene. The gene was organized in 24 exons that spanned 16 kb. Based on the gene sequence they identified two mutations in cattle.

At this time the  $\alpha$ -mannosidase knock-out mice provide the best available pharmacological model for investigative treatment of  $\alpha$ -mannosidosis to support regulatory submission. Acceptable models, which do not use live animals currently, do not exist.

#### Medical need for alpha-mannosidosis therapy

In light of the severe clinical manifestations resulting from the accumulation of mannose-rich oligosaccharides, the lack of effective treatment for alpha-mannosidosis is well recognised. At present, the major therapeutic options for treatment of the disease are bone marrow transplantation and enzyme replacement therapy.

#### Bone marrow transplantation

In 1996 Walkley et al. (Proc. Nat. Acad. Sci. 91: 2970-2974, 1994) published a paper on 3 kittens with mannosidosis that were treated with bone marrow transplantation (BMT) in 1991. In the 2 animals that were sacrificed a normalisation was seen, not only in the body, but more importantly, also in brain. The 3rd cat was well after 6 years. Normally, an untreated cat dies with 3-6 months. In 1987 a child with mannosidosis was treated with BMT (Will et al. Arch Dis Child 1987 Oct;62(10):1044-9). He died after 18 weeks due to procedure related complications. In brain little enzyme activity was found. This disappointing result could be explained by heavy immunosuppressive treatment before death, or that it takes time for the enzyme activity to increase in brain after BMT. The

WO 2005/094874

PCT/DK2005/000228

5

donor was the mother (who as carrier must be expected to have less than 50% enzyme activity) or it may be BMT in man has no effect on enzyme function in brain. Despite having variable outcomes the few attempts of bone marrow transplantation have thus indicated that successful engraftment can correct the clinical manifestations of alpha  
5 mannosidosis, at least in part. However, the challenge of reducing the serious procedure related complications when applying bone marrow transplantation i human therapy still remains to be defeated.

#### Enzyme replacement therapy

10 When lysosomal storage diseases were discovered, hopes were raised that this could be treated by enzyme substitution. Enzyme replacement therapy has proven efficient in Gaucher disease. When exogenous lysosomal glucocerebrosidase is injected into the patient, this enzyme is taken up by enzyme-deficient cells (Barton et al. N Engl J Med 324:1464-1470). Such uptake is regulated by certain receptors on the cell surface as for  
15 instance the mannose-6-phosphate receptor, which is nearly ubiquitous on the surface of cells and other receptors such as the asialoglycoprotein receptor and the mannose receptor, which are restricted to certain cell types such as cells of the monocyte/macrophage cell line and hepatocytes. The cellular uptake of the enzyme is therefore heavily dependent upon its glycosylation profile. If properly designed, the  
20 deficient enzyme could be replaced by regular injections of exogenous enzyme in the same manner as diabetic patients receive insulin. *In vitro* studies with the purified active lysosomal alpha-mannosidase added to the media of enzyme-deficient fibroblasts showed correction of the lysosomal substrate accumulation. *In vivo* treatment, on the other hand, has been hampered in part by the problem of producing the sufficient quantity of enzymes,  
25 and by complications resulting from immune reactions against the exogenous enzyme. Most importantly, however, special considerations apply in relation to lysosomal storage diseases with a major neurological component, such as alpha-mannosidosis, wherein the clinical manifestations are related to increased lysosomal storage within the central nervous system. Thus, enzyme replacement therapy has not proven effective against the  
30 acute neuronopathic variant of Gaucher disease (Prows et al. Am J Med Genet 71:16-21).

The delivery of therapeutic enzymes to the brain is prevented by the absence of transport of these large molecules through the blood-brain barrier. From the general notion that the blood brain barrier must be circumvented in order to see an effect of therapeutic agents in  
35 the brain, the use of a large diversity of delivery systems have been contemplated. These include invasive techniques such as osmotic opening of the blood brain barrier with for instance mannitol and non-invasive techniques such as receptor mediated endocytosis of chimeric enzymes. As enzyme replacement is expected to require administration of the enzyme on a regular basis, the use of invasive techniques should be avoided. Use of the

WO 2005/094874

PCT/DK2005/000228

6

non-invasive techniques, on the other hand, has not provided promising results. It has been contemplated that reduced storage in visceral organs and in the meninges could reduce the amount of oligosaccharides that is carried to the brain. Such considerations, however, are not considered to be applicable to lysosomal disorders in which the neurological damage is primary and severe (Neufeld, E. F. Enzyme replacement therapy, in "Lysosomal disorders of the brain (Platt, F. M. Walkley, S. V: eds Oxford University Press). Accordingly, the quest for an effective treatment of alpha -mannosidosis is still ongoing.

#### SUMMARY OF THE INVENTION

10 A first aspect of the present invention provides the use of a lysosomal alpha-mannosidase for the preparation of a medicament for reducing the intracellular levels of neutral mannose-rich oligosaccharides in cells within one or more regions of the central nervous system.

15 A second aspect pertains to a formulation of a lysosomal alpha-mannosidase for use as a medicament for reducing the intracellular levels of neutral mannose-rich oligosaccharides in cells within one or more regions of the central nervous system.

A third aspect of the invention pertains to a method of reducing the intracellular levels of neutral mannose-rich oligosaccharides in cells within one or more regions of the central nervous system of a subject, said method comprising administering to said subject a  
20 preparation of lysosomal alpha-mannosidase.

In preferred embodiments of both these aspects of the invention it is preferred that said medicament does not comprise a component with a known ability to target the central nervous system.

25 Other aspects of the invention pertain to methods of producing, isolating, and purifying an alpha-mannosidase as well as the use of an alpha-mannosidase, which is obtainable according to these methods.

#### DEFINITIONS AND TERMS

By "component" is meant, in broad terms, an element forming a part of the whole. More specifically, the term refers to a substance present in a mixture. Such a substance may be  
30 a compound defined as a material formed from elements chemically combined in definite proportions by mass. Examples of compounds inorganic compounds such as small molecular species, organic compounds such as carbohydrates including simple sugars and



WO 2005/094874

PCT/DK2005/000228

7

complex polysaccharide polymers, peptides, proteins, peptidomimetics, nucleic acids and antibodies

The term "central nervous system" refers to the brain, spinal cord, and spinal nerves. The  
5 central nervous system does not include the peripheral nerves in the arms, legs, muscles, and organs.

In the present context the term "brain" refers to that portion of the central nervous system that is located within the skull including its two (right and left) halves referred to as the  
10 hemispheres.

Also in the present context, extraneural cells such as cells in endothelia and epithelia of the choroid plexus are not to be considered parts of the central nervous system and the brain. Also the brain and central nervous system do not comprise the meninges.

15

The "choroid plexus" is a vascular proliferation or fringe of the tela choroidea in the third, fourth, and lateral cerebral ventricles; it secretes cerebrospinal fluid, thereby regulating to some degree the intraventricular pressure.

20 The term "meninges" refer to the three membranes that cover the brain and spinal cord. The outside meninx is called the dura mater, and is the most resilient of the three. The center layer is the pia mater, and the thin innermost layer is the arachnoid.

By "complex" or "conjugate" is meant a molecule or a group of molecules which are either  
25 held together by van der Waal's and/or electrostatic and/or by covalent bonds.

The term "exogenous" refers to a component which is introduced from or produced outside the organism or system; specifically, the term refers to components not synthesized within the organism or system.

30 The term "permeant" refers to a component, which is able to pass through a particular semipermeable membrane. Such a semipermeable membranes may be the blood-brain barrier and/or the foetal-maternal barrier.

The terms "blood-brain barrier" and "blood-cerebral barrier" refer to the barrier system  
35 separating the blood from the parenchyma of the central nervous system.

WO 2005/094874

PCT/DK2005/000228

8

The term "targeting" refers to the process of process of having proteins contain certain signals such that the proteins are directed specifically towards certain cellular locations, *e.g.*, the lysosome.

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#### DETAILED DESCRIPTION

The hallmark of  $\alpha$ -mannosidosis is the storage of neutral oligosaccharides in a wide variety of tissues (see Fig. 2, lane 1 and lane 2). These oligosaccharides contain 2-9 mannose residues and an N-acetylglucosamine residue at their reducing terminus and therefore  
10 result from the action of an endoglucosaminidase. The heavy involvement of the central nervous system is reflected in the clinical phenotype of alpha-mannosidosis ranging from severe infantile forms to milder juvenile forms with only moderate mental retardation. The rationale behind the present invention emerges from the surprising observation that repeated intravenous administration of recombinant alpha-mannosidase results in a  
15 marked reduction in the levels of stored neutral mannose-rich oligosaccharides in the brain.

In its broadest aspect, the present invention relates to a recombinant LAMAN polypeptide and to a nucleotide sequence encoding the LAMAN polypeptide, to an expression system capable of expressing the polypeptide as well as to pharmaceutical compositions  
20 comprising the polypeptide or part of it and to the use of the polypeptide for preventing or treating the development of symptoms related to alpha-mannosidosis caused by a deficiency, in a subject, of the Lysosomal alpha-mannosidase (LAMAN) enzyme.

In one main aspect, the invention provides the use of a lysosomal alpha-mannosidase for the preparation of a medicament for reducing the intracellular levels of neutral mannose-  
25 rich oligosaccharides in cells within one or more regions of the central nervous system, such as one or more regions within the brain. The neutral mannose-rich oligosaccharides contain two to nine mannose residues linked to a single N-acetylglucosamine residue.

Another main aspect of the invention pertains to a formulation of a lysosomal alpha-mannosidase for use as a medicament for reducing the intracellular levels of neutral  
30 mannose-rich oligosaccharides in cells within one or more regions of the central nervous system.

For both main aspects of the invention it is further preferred that the lysosomal alpha-mannosidase is selected from the group consisting of

- (a) the amino acid sequence of SEQ ID NO:1;
- 35 (b) a portion of the sequence in (a), which is enzymatically equivalent to recombinant human lysosomal alpha-mannosidase

WO 2005/094874

PCT/DK2005/000228

9

- (c) an amino acid sequence analogue having at least 75% sequence identity to any one of the sequences in (a) or (b) and at the same time being enzymatically equivalent to recombinant human lysosomal alpha-mannosidase.

It may be preferred that the degree of sequence identity between the nucleic acid sequence comprised within the cell according to the invention and SEQ ID NO: 1 is at least 80%, such as at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99%.

In the present context, an amino acid sequence analogue or a portion of an amino acid sequence, which is enzymatically equivalent to recombinant human lysosomal alpha-mannosidase, is a polypeptide, which has a specific activity of at least 5 U/mg when present at a purity of at least 90%. Purity is conveniently determined using conventional techniques such as SDS-PAGE. Specific activity may be determined in an enzyme assay as described in example 2 of the present application. In brief, the purified polypeptide is incubated with the substrate, p-nitrophenyl- $\alpha$ -mannopyranoside at 37°C for an appropriate amount of time before the reaction is stopped by the addition of 0.4 M glycine/NaOH pH 10.4. The amount of substrate converted may be determined by the change of absorbance at 405 nm ( $\epsilon=18500 \text{ M}^{-1} \text{ cm}^{-1}$ ).

In some embodiments the lysosomal alpha-mannosidase is formulated for systemic delivery. Formulations for systemic delivery may comprise a variety of pharmaceutically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. "Pharmaceutically acceptable" carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and for the present applications they are also present in concentrations which have no effect on the permeability of the blood-brain barrier. Included are buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecularweight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e. g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN, PLURONIC or polyethylene glycol (PEG).

WO 2005/094874

PCT/DK2005/000228

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Successful therapy of alpha-mannosidosis must effectively address the challenges posed by the central nervous system and the blood-brain barrier in particular as it prevents the entry of lysosomal enzymes into the brain. The anatomical component of the blood brain barrier consists of unique endothelial cells in the brain capillaries, having tight junctions without fenestrations and with few microvilli and few vesicles for fluid transport. Its physiologic component in part consists of enzymes unique to the brain endothelia and of active transport via carrier proteins.

Beyond the blood-brain barrier additional barriers are present. Perivascular spaces are patrolled by phagocytes that can sequester foreign compounds, and furthermore, layers of basal laminae, macromolecular meshes of protein lie between the vasculature and central nervous system parenchyma. After this, the *glia lamintan*, a sheath made up of the end feet of astroglia and microglia forms another physical barrier. Finally, solutes beyond the endothelial barrier are also subject to an outward bulk fluid flow through contiguous spaces. Interstitial fluid from the central nervous system parenchyma moves partly into the ventricular system and perivascular spaces and subsequently solutes are carried to the subarachnoid spaces from which soluble constituents return to the bloodstream. Thus multiple barriers exist that limit and regulate the entry into of elements from the blood stream into the brain.

It has been contemplated that the blood brain barrier problem may be solved with the development of brain drug targeting technology. This technology relies on the delivery of therapeutic proteins to the brain via access on endogenous transport systems localized within the brain capillary endothelium, which forms the blood-brain barrier in vivo.

In the following non-limiting examples of components with an ability to target or mediate targeting of the central nervous system and/or with a known ability to act as vehicles for the passage over the blood-brain barrier delivery and/or cellular membranes are given.

1) Peptides and proteins as vehicles for passage LAMAN to the target cells by passage over cell membranes and/or the BBB:

A number of studies in animals have shown that certain proteins and/or peptides may act as vehicles for passage of BBB. For instance proteins modified by the insulin fragment (Fukuta et al. *Pharmacol Res* 11: 1681-1688) or antibodies to the transferrin receptor (Friden et al. *Proc Natl Acad Sci USA* 88: 4771-4775) can pass the blood-brain barrier. Also proteins modified by coupling to polyamines (Poduslo and Curran. *J Neurochem* 66: 1599-1606) have been reported to pass the blood-brain barrier. In addition, Peptidomimetic monoclonal antibodies (MAb) undergo receptor-mediated transcytosis (RMT) through the BBB in vivo via transport on the endogenous BBB transferrin receptor (TfR) or insulin receptor (IR). Therapeutic proteins may be

WO 2005/094874

PCT/DK2005/000228

11

conjugated to the MAb transport vectors with either avidin-biotin technology or as fusion proteins.

2) Toxins as vehicles for passage LAMAN to the target cells by passage over cell

5 membranes and/or the BBB:

Different bacteria, plants and animals produce toxins. Toxins have many different targets such as the gut (enterotoxins), nerves or synapses (neurotoxins). Toxins can traverse cell membranes via receptor mediated processes and the embodiment of the present invention is to use toxins as vehicles to passage rhLAMAN to the target cells  
10 over cellular membranes and/or the BBB. The preferred target cells are cells in the CNS and/or the peripheral nervous system. It is to be understood that only the part of the toxin that is responsible for association with and translocation over cellular membranes is used.

15 One example of a toxin used as a vehicle is a bacterial toxin such as Diphtheria Toxin (DT), from the *Corynebacterium Diphtheriae*. Bacterial toxins exhibit a wide range of toxicities and they fall into groups by structure and function. The toxin binds to a target cell and enters the cell via a receptor, and is reduced to separate fragments. The processed toxin can be divided into the following 3 domains: The catalytic domain (C),  
20 the receptor domain (R), and the translocation domain (T).

The catalytic fragment and the receptor fragment of the toxin or fragments thereof are replaced by the LAMAN. This fusion protein can traverse cellular membranes and/or the BBB and thereby deliver the LAMAN to the target cells. One example of the engineering  
25 of a hybrid molecule could be by recombinant technology

Other examples of bacterial toxins to be used as vehicles are Clostridium Botulinum, Pseudomonas Exotoxin A produced by *Pseudomonas aeruginosa*, Cholera Toxin produced by *Vibrio cholerae*, and Pertussis Toxin produced by *Bordetella pertussis*.

30

Further examples of toxins used as vehicles are plant toxins selected from the list of the following plant toxins: cholinesterase inhibitors, protease inhibitors, amylase inhibitors, tannins, cyanogenic glycosides, goitrogens, lectin proteins, and lathyrrogens, pyrroldine alkaloids.

35

Yet further examples of toxins used as vehicle are toxins from shellfish (saxitoxin) and snakes (alpha-bungarotoxin). The skilled person may add further examples in light of the details and characteristics of the invention.

WO 2005/094874

PCT/DK2005/000228

12

3) Proteins and/or peptides isolated from bacteria or viruses as vehicles for passage rhLAMAN to the target cells by passage over cell membranes and/or the BBB:

- 5 The transacting element and/or transacting protein from bacteria or viruses can be used together with rhLAMAN to cross the BBB and/or cellular membranes.

Examples of bacteria are selected from the following list: *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Hemophilus influenzae*, *Staphylococcus* species, *Proteus* species,  
10 *Pseudomonas* species, *E. coli*, *Listeria monocytogenes*, *M. Tuberculosis*, *Neurospora*,  
*Spirochetes* *Borrelia burgdorferi* from *Ixodes ricinus*.

Examples of viruses are selected from the following list of virus families: *Parvoviridae*,  
*Papovaviridae*, *Adenoviridae*, *Herpesviridae*, *Poxviridae*, *Picornaviridae*, *Reoviridae*,  
15 *Togaviridae*, *Arenaviridae*, *Coronaviridae*, *Retroviridae*, *Bunyaviridae*,  
*Orthomyxoviridae*, *Paramyxoviridae*, and *Rhabdoviridae*. Relevant examples of viruses  
selected from the list mentioned above are e.g. Measles virus, Papova virus, and JC  
virus.

20 4) Cell mediated delivery systems

A cell-mediated approach to therapy may address storage disorders in more than one  
fundamental way. The first is to replace or compensate for defective cell populations  
with normal equivalents. A second way to obtain therapeutic benefit can be through  
cross correction. Appropriate cell types offer the ability to not only cross the endothelial  
25 barrier from the blood but to actively migrate into the parenchyma and take up  
residence within the milieu of the diseased cells. The preferred human cell is selected  
from human monocytes, human fibroblasts, and human lymphocytes.

5) Disturbing the Blood-Brain Barrier

30 Another invasive strategy for enhanced CNS drug delivery involves the systemic  
administration of drugs in conjunction with transient BBB disruption (BBBD).  
Theoretically, with the BBB weakened, systemically administered drugs can undergo  
enhanced extravasation rates in the cerebral endothelium, leading to increased  
parenchymal drug concentrations. A variety of techniques that transiently disrupt the  
35 BBB have been investigated; however, albeit physiologically interesting, many are  
unacceptably toxic and therefore not clinically useful. These include the infusion of  
solvents such as dimethyl sulfoxide or ethanol and metals such as aluminium; X-  
irradiation; and the induction of pathological conditions including hypertension,  
hypercapnia, hypoxia or Ischemia.

WO 2005/094874

PCT/DK2005/000228

13

A somewhat safer technique involves the systemic delivery of the convulsant drug, metrazol, which transiently increases the BBB permeability while causing seizures. Concurrent administration of the anticonvulsant pentobarbital blocks seizing while  
5 allowing BBBD to persist. The BBB can also be compromised by the systemic administration of several antineoplastic agents including VP-16, cisplatin, hydroxylurea, flurouracil and etoposide.

#### Osmotic Blood-Brain Barrier Disruption

10 Intracarotid injection of an inert hypertonic solution such as mannitol or arabinose has been employed to initiate endothelial cell shrinkage and opening of BBB tight junctions for a period of a few hours, and this permits delivery of antineoplastic agents to the brain. Osmotic disruption has been tested as a strategy for the delivery of macromolecular drugs such as monoclonal antibodies, nanoparticles and viruses.  
15 However, the procedure breaks down the self-defense mechanism of the brain and levels it vulnerable to damage or infection from all circulating chemicals or toxins. The risk factors include, the passage of plasma proteins, the altered glucose uptake, the expression of heat shock proteins, microembolism or abnormal neuronal function.

20 While a large number of techniques have thus been developed for facilitating the delivery of compounds over the blood brain barrier many of them show limited success in addressing the other barriers mentioned above. Their applicability in the treatment of alpha-mannosidosis therefore appears limited.

In contrast, a very important aspect of the invention however pertains to a scheme for the  
25 reduction of the intralysosomal accumulation of neutral mannose-rich oligosaccharides within the central nervous system based on a technique, which is not dependent on means for mediate transport across and/or penetrate and/or disrupt the blood-brain barrier. In preferred embodiments of the invention the above described approached to accessing the central nervous system are therefore disclaimed. In a preferred embodiments of the  
30 invention the medicament and the formulation according to the invention do not comprise a component with a known ability to target or mediate targeting of cells within the central nervous system and/or a component with a known ability to act as vehicle for the passage over the blood-brain barrier.

Expressed in more exact terms said medicament or formulation does not comprise a  
35 component with a known ability to target the neuronal and/or glial cells of the brain.

WO 2005/094874

PCT/DK2005/000228

14

Preferred embodiments thus pertain to the use of lysosomal alpha-mannosidase and to a formulation of lysosomal alpha-mannosidase, wherein said medicament or formulation does not comprise lysosomal alpha-mannosidase in a form, which is capable of permeating the central nervous system/blood brain barrier. Accordingly, it is also preferred that the

5 lysosomal alpha-mannosidase has not been modified in order to obtain carrier-mediated transport across the blood-brain barrier and that the lysosomal alpha-mannosidase has not been formulated so as to obtain disruption of the blood-brain barrier.

In equally preferred embodiments the medicament or formulation comprises a lysosomal alpha-mannosidase and does not comprise

- 10 a) a vehicle or vector, such as a peptide or polypeptide, for delivery of lysosomal alpha-mannosidase into the central nervous system, and
- b) a component with a known ability to cause opening or disruption of the blood brain barrier, and
- c) an intact cell

15 More specifically, it is an object of the present invention to provide a formulation of a lysosomal alpha-mannosidase as well as the use of a lysosomal alpha-mannosidase for the preparation of a medicament, wherein said formulation or medicament does not comprise a vehicle selected from the group consisting of exogenous peptides and proteins, including the insulin fragment and peptides and proteins derived from virus or bacteria, polyamines,

20 lipophilic moieties including phospholipids, antibodies or antibody fragments such as transferrin receptor antibodies and toxins.

Also, the invention pertains to the use of lysosomal alpha mannosidase and to a formulation of lysosomal alpha mannosidase wherein said preparation of a medicament or said formulation does not comprise modification of said lysosomal alpha-mannosidase by

25 attachment of polyamines.

The medicament according to the invention may comprise or consist of a lysosomal alpha-mannosidase, which is formulated in an isotonic solution. Likewise the formulation of a lysosomal alpha-mannosidase according to the invention may comprise or consist of lysosomal alpha-mannosidase in an isotonic solution. The term "isotonic solution" refers

30 conventionally to a solution having the same osmotic pressure as blood. In the context of the present invention the solution may be but is not limited to a human isotonic solution. Presently preferred are formulation buffers comprising or consisting of 3.10-3.50 mM  $\text{Na}_2\text{HPO}_4$ , 0.4-0.6 mM  $\text{NaH}_2\text{PO}_4$ , 25-30 mM Glycine, 220-250 mM Mannitol, and water for injection. Equally preferred are formulation buffers comprising or consisting of 4.0-5.0

35 mM Sodium Citrate, 0.3-0.8 mM Citric Acid, 200-250 mM Mannitol, 3.0-5.0 mM Tween 80, and water for injection. Alternatively, the lysosomal alpha-mannosidase may be formulated in Phosphate buffered saline (0.01 M Phosphate buffer + 0.145 M NaCl, pH=7.2).



WO 2005/094874

PCT/DK2005/000228

15

As mentioned above, the use of intact cells may provide means for delivery of substances across the blood-brain barrier. The present invention, however relates to a medicament or formulation which does not comprise an intact cell.

Accordingly, a further preferred embodiment of the invention pertains to a formulation of lysosomal alpha mannosidase as well as to the use of lysosomal alpha mannosidase, wherein said formulation or preparation of a medicament does not involve the use of a cell-mediated delivery system. In particular the present invention does not rely on the use of an intact cell selected from the group consisting of a transduced autologous cell, such as a transduced fibroblast or a peripheral blood lymphocyte and polymer encapsulated cell line capable of secreting alpha-mannosidase into the cerebrospinal fluid.

It is to be expected that the administration of lysosomal alpha-mannosidase will result in decreased accumulation of neutral oligosaccharides in a large variety of cells within the central nervous system. In preferred embodiments of the invention, however, said cells within one or more regions of the central nervous system comprise neuronal cells and/or glial cells. In addition, said cells may comprise extraneural cells present within the central nervous system.

The brain comprises four major parts, the cerebrum, the diencephalon, the brainstem and the cerebellum. It is to be understood that administration of alpha-mannosidase affects the storage of oligosaccharides in one or more of these parts. In further preferred embodiments of the invention said one or more regions the central nervous system comprise one or more regions within the cerebrum, diencephalon, brainstem and/or cerebellum.

It is likely that reduction in the accumulation of neutral mannose-rich oligosaccharides within certain specific regions of the four above-mentioned brain parts is of particular relevance with respect to reducing the clinical manifestations of alpha-mannosidosis. In more particular embodiments of the invention the accumulation of oligosaccharides is reduced within one or more regions within the cerebrum being selected from the group comprising one or more regions within the cerebral cortex, one or more regions within the subcortical nuclei, and one or more regions within the limbic system.

A number of sensory and/or motory functions have been ascribed to various specific regions within the cerebral cortex. Within the Frontal Lobe, which is generally generally motor in function, the Precentral Gyrus (Primary Motor Cortex) is responsible for control of fine movement, the Motor Association Cortex is responsible for Integration of information for complex movements, the Speech-Motor Cortex is involved in production of speech;

WO 2005/094874

PCT/DK2005/000228

16

(Broca's area), and the Pre-Frontal Cortex is involved in decision making; direction of attention.

Within the Parietal Lobe, which is generally sensory in function, the Postcentral Gyrus (Primary Somatosensory Cortex) is involved in perception of sensation from skin

- 5 (Cutaneous) and muscle (proprioceptive), the Sensory Association Cortex is involved in integration of sensory signals; direction of sensory attention, the Left Association Cortex is involved in production of meaning to language; (Wernicke's area), and the Right Association Cortex is involved in production of meaning to spatial relationships

- 10 Within the Occipital Lobe, which is generally sensory in function, the Visual Cortex is involved in perception of visual sensation the Visual Association Cortex is involved in perception of visual content and meaning

Within the Temporal Lobe, which is generally sensory in function, the Primary Auditory

- 15 Cortex is involved in perception of auditory sensation, the Auditory Association Cortex is involved in perception of auditory content and meaning; it extends into parietal cortex Gustatory and Olfactory Cortex which are involved in perception of gustatory and olfactory sensation, the Insular Cortex located at the deep extent of lateral sulcus is involved in providing knowledge about outcome of events.

20

Other regions include the Parahippocampal Gyrus, which overlies the hippocampus, the Hippocampus, which is involved in formation of long-term memory and the Amygdala, which is involved with feeling of emotion.

- 25 In even more particular embodiments of the invention, the levels of oligosaccharides are reduced in one or more regions within the cerebral cortex being selected from the group comprising the frontal lobe, the parietal Lobe, the temporal lobe and the occipital Lobe.

In other particular embodiments of the invention, the levels of oligosaccharides are reduced in one or more regions within the diencephalon being selected from the group comprising the thalamus and the hypothalamus.

- 30 The thalamus is a component of the diencephalon buried deep within the core of the brain. Richly and reciprocally connected with cortical structures, thalamic nuclei function both to relay and modulate the transmission of information throughout the brain.

The thalamus comprises the following main parts: The anterior nuclear group, the midline

- 35 nuclear group, the medial dorsal nucleus, the intralaminar nuclear group, the lateral nuclear group, the ventral nuclear group, the geniculate bodies, the posterior nuclear

WO 2005/094874

PCT/DK2005/000228

17

complex, the thalamic reticular nucleus, the thalamic fiber tracts, and the stratum zonale of thalamus.

The limbic system is a group of brain structures that are involved in various emotions such as aggression, fear, pleasure and also in the formation of memory. The limbic system also affects the endocrine system and the autonomic nervous system. It consists of several structures located around the thalamus, just under the cerebrum: The hippocampus, which is involved in the formation of long-term memory, the amygdala, which is involved in aggression and fear, the cingulate gyrus, the fornicate gyrus, the archicortex and the hypothalamus, which controls the autonomic nervous system and regulates blood pressure, heart rate, hunger, thirst, and sexual arousal. Connected to the pituitary gland and thus regulates the endocrine system.

Neurological functions located in the brainstem include those necessary for survival (breathing, digestion, heart rate, blood pressure) and for arousal (being awake and alert). Most of the cranial nerves come from the brainstem. The brainstem is the pathway for all fiber tracts passing up and down from peripheral nerves and spinal cord to the highest parts of the brain. In additional particular embodiments of the invention the levels of oligosaccharides are reduced in one or more regions within the brainstem being selected from the group comprising the midbrain, the pons and the medulla oblongata.

The medulla oblongata functions primarily as a relay station for the crossing of motor tracts between the spinal cord and the brain. It also contains the respiratory, vasomotor and cardiac centers, as well as many mechanisms for controlling reflex activities such as coughing, gagging, swallowing and vomiting. The midbrain serves as the nerve pathway of the cerebral hemispheres and contains auditory and visual reflex centers. The pons is a bridge-like structure, which links different parts of the brain and serves as a relay station from the medulla to the higher cortical structures of the brain. It contains the respiratory center.

30

The function of the cerebellum, which includes the cerebellar cortex, is to facilitate the performance of movements by coordinating the action of the various participating muscle groups. In other particular embodiments of the invention, the levels of oligosaccharides are reduced in one or more regions within the cerebellum comprising the cerebellar cortex.

A primary advantage of the use of recombinant lysosomal alpha-mannosidase according to the present invention relates to fact that effects of the treatment seen in brain may combine with effects in cells that are not part of the central nervous system. Thereby, the administration of exogenous lysosomal alpha-mannosidase is likely to correct most if not all of the clinical manifestations of alpha-mannosidosis. In preferred embodiments, the

WO 2005/094874

PCT/DK2005/000228

18

invention thus pertains to the use of a medicament or formulation which further reduces the levels of neutral mannose-rich oligosaccharides in cells of neurological tissues, which are not within the central nervous system. In this embodiment of the invention, said medicament or formulation further comprises a component, which is capable of targeting  
5 cells of neurological tissues, which are not within the central nervous system. In particular the use of this medicament or formulation results in the correction of lysosomal storage in cells, which are not within the central nervous system, comprising the most prominent cells of the peripheral nervous system. These comprise neuronal cells and/or Schwann cells.

10 The clinical manifestations of alpha-mannosidosis may be partly alleviated by the isolated effects of replacing the deficient alpha-mannosidase in visceral tissues. However, a central part of the concept, which forms the basis of the present invention, relates to the fact that the beneficial effects of the therapy on lysosomal storage in the central nervous system is secondary to and/or dependent on a reduction in the lysosomal storage in tissues outside  
15 the central nervous system. Accordingly, it is an important characteristic of the invention that said medicament is also used to obtain a reduction in the levels of neutral mannose-rich oligosaccharides in cells within non-neurological tissues and that the medicament comprises a component capable of targeting cells within such tissues.

In this context, the effects of exogenous alpha-mannosidase in viscereal tissues are  
20 believed to be of primary importance. According to a preferred embodiment of the invention the medicament or formulation therefore comprises a component, which is capable of targeting neurological and/or non-neurological tissues cells outside the central nervous system. In an even more preferred embodiment said neurological and/or non-neurological tissues outside the central nervous system comprise visceral tissues selected  
25 from the group comprising liver, spleen, kidney and heart.

It is implicated that the exogenous alpha-mannosidase can be delivered to target cells in relevant tissues outside the central nervous system by use of for instance receptor mediated endocytosis. Preferably, the alpha-mannosidase is administered over cellular membranes of such target cells taking advantage of a mannose- or mannose-P-6-receptor-  
30 mediated uptake. Accordingly, the above mentioned factor possessing targeting abilities is preferably mannose-6-phosphate.

It is contemplated that any mammalian alpha-mannosidase may be used for replacing the deficient enzyme in a subject. However, when the subject is a human the use of a human lysosomal alpha-mannosidase will be preferred in order to, for instance, minimise the  
35 occurrence of antigenic reactions.. Accordingly, preferred embodiments of the invention pertains to the use of a lysosomal alpha mannosidase, which is a human lysosomal alpha . mannosidase.

WO 2005/094874

PCT/DK2005/000228

19

In a most preferred embodiment, the lysosomal alpha mannosidase comprises the amino acid sequence of SEQ ID NO.: 1.

For practical and economical reasons it is preferred that the alpha-mannosidase is produced recombinantly. Furthermore, it is to be expected that for a lysosomal hydrolase  
5 purified from tissue that the enzyme has a low content of mannose-6-phosphate. Thus, by recombinant production it will also be possible to obtain a preparation of the enzyme wherein a large fraction contains mannose-6-phosphate. Recombinant production may be achieved after transfection of a cell using a nucleic acid sequence comprising the sequence of SEQ ID NO: 2.

10 The alpha-mannosidase is preferably made in a mammalian cell system in order to a provide a glycosylation profile, which ensures efficient receptor mediated uptake in cells of for instance visceral organs of the body. In particular, it has been found that production of the enzyme in CHO, COS or BHK cells ensures adequate post-translational modification of the enzyme by addition of mannose-6-phosphate residues. In addition a correct sialylation  
15 profile is obtained. Correct sialylation is known to be important in order to prevent uptake by the liver, because of exposed galactose residues.

In even more preferred embodiments the mammalian cell system is therefore selected from the group comprising CHO, COS cells or BHK cells (Stein et al. J Biol Chem.1989, 264, 1252-1259). It may further be preferred that the mammalian cell system is a human  
20 fibroblast cell line.

In a most preferred embodiment, the mammalian cell system is a CHO cell line.

It follows that equally preferred embodiments of the invention pertains to the use of a preparation of lysosomal alpha-mannosidase wherein a fraction of said preparation consists of lysosomal alpha mannosidase having one or more N-linked oligosaccharides  
25 carrying mannose 6-phosphate groups.

It is further preferred that a fraction of a preparation of said lysosomal alpha-mannosidase is capable of binding to mannose 6-phosphate receptors.

The ability of the enzyme to bind to mannose-6-phosphate receptors may be determined in an *in vitro* assay as described in example 1 of the present application. Here, binding of  
30 the enzyme to a MPR affinity 300 Matrix provides a measure of its ability to bind to mannose-6-phosphate receptors. In a preferred embodiment of the invention binding of the enzyme to mannose-6-phosphate receptors occurs *in vitro*.

In more preferred embodiments of the invention this fraction corresponds to from 1 to 75% of the activity of a preparation of lysosomal alpha-mannosidase, such as from 2 to

WO 2005/094874

PCT/DK2005/000228

20

70%, such as from 5 to 60%, such as from 10 to 50% such as from 15 to 45%, such as from 20 to 40%, such as from 30 to 35%.

Accordingly, it is preferred that the lysosomal alpha-mannosidase has a content of mannose 6-phosphate residues allowing mannose 6-phosphate dependent binding of from 5 2 to 100%, 5 to 95%, 10 to 90%, 20 to 80%, 30 to 70% or 40 to 60% of the amount of enzyme to a Man-6-P-receptor matrix. At present, the degree of phosphorylation has been analysed in several batches of enzyme and, typically, from 30 to 45% of the enzyme is phosphorylated and binds the affinity matrix.

10 It is further preferred that a fraction constituting from 2 – 100%, 5 – 90%, 10 – 80%, 20 – 75%, 30 – 70%, 35 – 65% or 40 – 60% of the amount of said lysosomal alpha-mannosidase binds to the Man-6-P-receptor with high affinity. Theoretically, two mannose 6-phosphate groups must be positioned close to each other in order for the enzyme to bind a Man-6-P-receptor with high affinity. Recent observations suggest that the distance  
15 between the phosphorylated mannose residues must be 40 Å or less in order to obtain high affinity binding. In the human lysosomal alpha-mannosidase according to SEQ ID NO: 1 the two mannose 6-phosphate residues may be situated at the asparagines residues in positions 367 and 766. Accordingly, it is preferred that the formulation and medicament according to the present invention comprises lysosomal alpha-mannosidase, a fraction of  
20 which carries mannose 6-phosphate groups at both of these asparagine residues.

It is to be understood that various different means of administration can be employed for delivering the enzyme. These include parenteral including intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion. Since administration is likely to be repeated on a regular basis the use of simple techniques for the administration  
25 is preferred. In a currently most preferred embodiment of the invention, alpha mannosidase is used for the preparation of a medicament, which is for intravenous administration. In particular, administration by injection directly into the central nervous system is considered not to be suitable since administration via this route cannot be performed by a lay-person.

30 It will of course be possible to co-administer the medicament, which is prepared by the use of the alpha-mannosidase according to the invention, or the alpha-mannosidase formulation according to the invention with a composition comprising a component with a known ability to target or mediate targeting of the central nervous system. In a much-preferred embodiment of the present invention such co-administration is neither necessary  
35 nor desired and is not to be included in the treatment scheme. This embodiment pertains to the use according to the invention, wherein said medicament or formulation is for administration without supplementary administration of a composition comprising a component with a known ability to target the central nervous system.

WO 2005/094874

PCT/DK2005/000228

21

It appears that the medicament or formulation is to be administered in doses, wherein the amount of lysosomal alpha-mannosidase is sufficient to correct the defects in lysosomal storage. It appears from previous studies on related lysosomal disorders, that a complete substitution of the deficient enzyme, so that levels corresponding to those seen in healthy individuals are reached, may not be necessary. In order to minimise the cost of the therapy and possibly also reduce putative adverse effects the aim should be to use as small amounts of the enzyme as possible. In addition, it appears possible to "tailor" the treatment including the amounts administered and the frequency of administration according to the profile of the individual subject. Therefore, in preferred embodiments of the present invention, the medicament is administered in an amount, which is within the range of 1 to 400 mU lysosomal alpha-mannosidase per gram body weight of the subject to be treated, such as within the range of 2 to 350, 5 to 300, 7,5 to 200, 10 to 200, 15 to 150, 20 to 100, 25 to 75, 30 to 60, 35 to 50 or 40 to 45 mU lysosomal alpha-mannosidase per gram body weight,

In a currently more preferred embodiment of the invention the medicament or the formulation is administered in an amount of 250 mU lysosomal alpha-mannosidase per gram body weight. In an equally preferred embodiment of the invention, the medicament or formulation is administered in an amount of 50 mU lysosomal alpha-mannosidase per gram body weight.

The specific activity of the lysosomal alpha-mannosidase may be a crucial factor influencing the ability of the wherein said lysosomal alpha-mannosidase has a specific activity of 5 - 100 U/mg, such as from 10 - 90 U/mg, 15 - 80 U/mg, 20 - 70 U/mg, 25 - 65 U/mg, 30 - 60 U/mg or 35 - 55 U/mg.

In the enzyme preparation used in the present invention, a fraction of the amount of lysosomal alpha-mannosidase is present in the form of a 260 kDa dimer composed of 130 kDa monomers of an amino acid sequence having at least 75%, 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 1. When analysed under reducing conditions, however, a variable fraction of the amount of enzyme appears to have been processed into the 70 and 55 kDa forms. This indicates that a fraction of the amount of the lysosomal alpha-mannosidase has been partly cleaved but is still held together in the 260 kDa form by disulfide bridges. Both the 130 kDa form and the processed forms are active, but it is preferred that a large fraction of the amount of enzyme is present as dimers of the 130 kDa precursor form when administered to the subject. Preferably, at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% is present as a dimer of the 130 kDa form. When the enzyme is subsequently taken up into the lysosomes it is processed into the 15, 42 and 70 kDa peptides. The 70 kDa peptide is further processed to three smaller peptides that are still kept together by disulfide bridges.

WO 2005/094874

PCT/DK2005/000228

22

As enzyme replacement therapy is only expected to generate transient effects, it further appears that the medicament or formulation may be administered in a frequency ranging from several daily injections to injections once or twice every week or month. In preferred  
5 embodiments the medicament or formulation may be administered in a frequency corresponding to 1 to 5 daily injections, 1 to 5 weekly injections, 1 to 5 injections every 2 weeks or 1 to 5 injections every month. Alternatively, the frequency may correspond to one injection every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days.

In equally preferred embodiments the medicament or formulation is administered by  
10 repeated intravenous injection on a daily basis, by repeated intravenous injection every 2, 3, 4, 5, or 6 days, or by repeated intravenous injection on a weekly, bi-weekly or monthly basis.

In a currently preferred embodiment of the invention the amount of lysosomal alpha-mannosidase is administered once every 3 to 4 days.

15 Administration of the formulation or the medicament according to the present invention by intravenous injection leads to only insignificant increases in the amount of lysosomal alpha-mannosidase present within the central nervous system. While not being bound by this theory it appears that the reduced levels of neutral oligosaccharides may be secondary to and a direct or indirect result of the increased levels of enzyme in the peripheral tissue  
20 and visceral organs. Since the lysosomal alpha-mannosidase appears to be rapidly cleared from the circulation, it appears critical that the amounts of enzyme administered are such that the levels of circulating enzyme remain adequately increased for an amount of time allowing for cellular uptake of appropriate amounts of enzyme via the Man-6-P receptor or, possibly, also via other presently unknown transport mechanisms. In addition, levels of  
25 enzyme within the visceral organs and the peripheral tissue must remain adequately increased for an amount of time sufficient to cause a reduction in the neutral sulfatides not only within the organs or tissue but also within the central nervous system. Accordingly it is preferred that the amount of lysosomal alpha-mannosidase administered is such that:

- a) effective levels of the enzyme are sustained in circulation for not less than 5 – 30  
30 minutes such as not less than 5, 10, 15, 20, 25, or 30 minutes, and/or
- b) effective levels of the enzyme are sustained in visceral organs, including the kidney, spleen and heart, for not less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, or 24 hours, and/or
- c) effective levels of the enzyme are sustained in the liver for not less than 6, 12 or 24  
35 hours or 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 days,

subsequent to injection, such as intravenous injection, of said medicament or formulation.



WO 2005/094874

PCT/DK2005/000228

23

In the present context the term "effective levels" refer to levels of lysosomal alpha-mannosidase that are increased by at least two fold, at least 5 fold, at least 10 fold, at least 20 fold or at least 50 fold compared to the enzyme levels seen in serum or organ extracts from untreated subjects when analysed in an enzyme assay as described in

5 example 2 of the present application.

Preferably, the enzyme once administered to said subject will lead to a reduction in the levels of intralysosomal oligosaccharides preferably corresponding to 20%, more preferably 30%, even more preferably 40%, still more preferably 50%, yet more preferably 60%, most preferably more than 70%.

10 In particular, the amount of lysosomal alpha-mannosidase administered is preferably such that:

- a) the amount of neutral oligosaccharides in visceral organs including the liver, spleen, kidney and/or heart is reduced by 10 – 80%, 15 – 85%, 20 – 90%, 25 – 95% or 30 – 100%, such as at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60,  
15 65, 70, 75, 80, 85, 90 or 95% over 2, 4, 6, 8, 10, 12 18 or 24 hours, as compared to the levels before treatment or the levels seen in untreated subjects, and/or
- b) b) the amount of neutral oligosaccharides in visceral organs including the liver, spleen kidney and/or heart is reduced by 40 – 80%, 45 – 85%, 50 – 90%, 55 – 95% or 60 – 100%, such as at least by 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90  
20 or 95% over a period of 1 – 4, 2 – 6 or 4 – 8 days , and/or
- c) c) the storage of neutral oligosaccharides in the liver is reduced over a period of 1 to 12, 2 – 10 or 4 - 8 days as determined by a reduced presence of storage vacuoles within the sinus endothelial cells, Kupffer cells and hepatocytes subsequent to injection, such as intravenous injection, of said medicament.

25

Central to the present invention is the observation that a reduction in the levels of neutral oligosaccharides in the central nervous system is seen only after repeated administration of lysosomal alpha-mannosidase. Therefore, it may be preferred that the amount of lysosomal alpha-mannosidase administered is such that:

- 30 a) effective levels of the enzyme are sustained in circulation, and/or
- b) effective levels of the enzyme are sustained in visceral organs, including the kidney, spleen and heart, and/or
- c) effective levels of the enzyme are sustained in the liver,  
by repeated injection, such as intravenous injection, of the medicament on a daily basis,  
35 by repeated injection, such as intravenous injection, every 2, 3, 4, 5, or 6 days, or by  
repeated injection, such as intravenous injection, on a weekly, bi-weekly or monthly basis.

It may be equally preferred that the amount of lysosomal alpha-mannosidase administered is such that

WO 2005/094874

PCT/DK2005/000228

24

- a) the storage of neutral oligosaccharides in kidney and heart is fully corrected or fully corrected at least over a period of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 days, and/or
- 5 b) the amount of neutral oligosaccharides in the spleen is reduced by at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100% or so reduced at least over a period of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 days, and/or
- 10 c) the storage of neutral oligosaccharides in the liver and the kidney is reduced or reduced over a period of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 days as determined by a reduced presence of storage vacuoles within the sinus endothelial cells, Kupffer cells and hepatocytes of the liver and in the tubular epithelia of the kidney

by repeated injection, such as intravenous injection, of the medicament on a daily basis, by repeated injection, such as intravenous injection, every 2, 3, 4, 5, or 6 days, or by repeated injection, such as intravenous injection, on a weekly, bi-weekly or monthly basis.

15

The reduction of stored oligosaccharides in the brain may result, at least in part, from the clearance of storage material from brain endothelia and from the meninges, which may improve the microcirculation and the flow of the cerebrospinal fluid. A much preferred embodiment of the present invention thus pertains to the use of lysosomal alpha-

20 mannosidase for the preparation of a medicament for reducing the intracellular levels of neutral mannose-rich oligosaccharides in cells within one or more regions of the brain endothelia and/or the meninges.

Another main aspect of the invention pertains to a method of reducing the intracellular levels of neutral mannose-rich oligosaccharides in cells within one or more regions of the

25 central nervous system of a subject, said method comprising administering to said subject a medicament or a formulation comprising a preparation of lysosomal alpha-mannosidase,

It is to be understood that the medicament or formulation may have the characteristics describe above. Thus, in a preferred embodiment of this aspect of the invention, said medicament or formulation does not comprise a component with a known ability to target

30 or mediate targeting of the central nervous system and/or a component with a known ability to act as vehicle for the passage over the blood-brain barrier.

Again it is preferred that the method comprises administering said medicament or formulation of alpha-mannosidase by a route other than Intracerebroventricular, spinal, intrathecal or Intracranial administration.

35 Particularly preferred is administration of the medicament or formulation by intravenous or subcutaneous injection.

WO 2005/094874

PCT/DK2005/000228

25

Co-administration of the medicament or formulation according to the present invention with a component with a known ability to target or mediate targeting of the central nervous system and/or a component with a known ability to act as vehicle for the passage over the blood-brain barrier is neither necessary nor desired and is not to be included in the treatment scheme. Therefore, a preferred embodiment of this aspect of the Invention pertains to a method according to the invention, wherein said medicament or formulation is administered without supplementary administration or co-administration of a composition comprising a component with a known ability to target the central nervous system. It is thus preferred that the administration of said medicament or formulation is not supplemented by the administration of a composition comprising a component with a known ability to target the central nervous system.

According to a recent publication, intrathecal injection, that is injection directly into the cerebrospinal fluid) of recombinant human alpha-L-iduronidase (rhIDU) can reduce carbohydrate storage in brain tissue in a canine model of mucopolysaccharidosis (MPL) (Kakkis, 2003). While intravenous or subcutaneous administration of the medicament or formulation according to the present invention is by far preferred, the observations of Kakkis suggest that such administration may at least in some cases be combined with other treatments, including other routes of administration. Thus, the present invention may provide methods of treatment which comprise:

- (a) administration of a medicament or formulation according to the invention intravenously in order to obtain a reduction in neutral mannose-rich oligosaccharide levels in target cells within the peripheral nervous system, optionally as an adjunct to treatment by bonemarrow transplantation; and/or
- (b) administration of a medicament or formulation according to the invention intravenously in order to obtain a reduction in neutral mannose-rich oligosaccharide levels in target cells both within the peripheral and central nervous system; and/or
- (c) administration of a medicament or formulation according to the invention intravenously in order to obtain a reduction in neutral mannose-rich oligosaccharide levels in target cells within the peripheral nervous system, optionally in combination with intrathecal injection of a pharmaceutical composition or formulation according to the invention in order to obtain a reduction in neutral mannose-rich oligosaccharide levels in target cells within the central nervous system.

The invention may thus provide the use of a lysosomal alpha-mannosidase for the preparation of a medicament for intravenous administration as well as for administration by intrathecal or spinal injection, wherein the combined intravenous and intrathecal administration results in a reduction in the galactosyl sulphatide levels in target cells within the peripheral nervous system and in target cells within the central nervous system in a

WO 2005/094874

PCT/DK2005/000228

26

subject. Likewise, the invention may provide a formulation of lysosomal alpha-mannosidase for intravenous as well as intrathecal or spinal injection.

Further, the invention may provide the use of a lysosomal alpha-mannosidase for the  
5 preparation of a medicament for intravenous administration and a formulation comprising a lysosomal alpha-mannosidase for intravenous administration, where intravenous administration of the medicament or formulation results in a reduction in the galactosyl sulphatide levels in target cells within the peripheral nervous system and in target cells within the central nervous system.

10

In yet another aspect, the invention provides a method for the preparation of recombinant human alphanmannosidase, the method comprising a) introducing, into a suitable vector, a nucleic acid fragment comprising a DNA fragment which codes for the amino acid sequence shown SEQ ID NO.:1; b) transforming a cell with the vector obtained in step a);  
15 c) culturing the transformed host cell under conditions facilitating expression of the nucleic acid sequence; d) recovering the expression product from the culture. The method may further comprise a series of one or more purification steps.

In particular, the invention relates to a method wherein the nucleic acid fragment  
20 comprises the 3066 basepair EcoRI - XbaI fragment of the DNA fragment shown in SEQ ID NO 2.

The production of recombinant alpha-mannosidase, which is mannose-6-P comprises one or several or all of the following general steps (an outline):

25

#### A. Synthesis of rhLAMAN

Cloning of specific rhLAMAN

#### B. Transfection

30 2-10 µg rhLAMAN hybrid vector DNA is used for transfection by phosphate precipitate/ glycerol shock methodology, into mammalian cells (e.g. CHO, COS cells or BHK cells). Transfection might also be done with an electric shock methodology.

#### C. Expression of rhLAMAN

35 Synthesis of the active protein and the mannose-6-P modification of said protein is done during the expression in the mammalian cell system.

#### D. Purification of rhLAMAN

WO 2005/094874

PCT/DK2005/000228

27

recombinant human alpha-mannosidase is purified using a 6 step procedure - see example 1.

E. Test system for mannose-6-P receptor mediated uptake

- 5 The ability of produced recombinant alpha-mannosidase to be active in a mannose-6-P receptor mediated uptake is tested by incubating alpha-mannosidase with normal fibroblasts or fibroblasts from alpha-mannosidosis patients. Uptake into cells is assayed by increased alpha-mannosidase activity.
- 10 In a currently preferred embodiment of the invention the alpha-mannosidase is produced in a mammalian cell system selected from the group comprising BHK, COS and CHO cells. One of the critical parameters of the method according to the invention is the glycosylation profile of the alpha-mannosidase in the final preparation. The glycosylation profile is a major determinant of the corrective activity of the enzyme in vivo via its effects on
- 15 receptor mediated endocytosis and clearance of the enzyme from the blood stream. When using the above mentioned preferred mammalian cell systems it has been found that alpha-mannosidosis with an appropriate glycosylation profile is produced; in particular use of CHO cells leads to the production of alpha-mannosidase with an adequate content of mannose-6-phosphate. In the examples of the present application the ability of the alpha-
- 20 mannosidase to bind to an MPR 300 column provides a measure of its content of mannose-6-phosphate. The mannose-6-phosphate containing alpha-mannosidase is secreted into the medium and purification of the enzyme may be facilitated by the use of ammonium salts (NH<sub>4</sub>Cl) in the fermentation step.
- 25 Currently, a factor, which must be taken into consideration when isolating and purifying the enzyme, is the occurrence of dephosphorylation during isolation. While preparation of human alpha-mannosidase have been obtained in which a fraction corresponding to 40% or more of the enzyme activity was able to bind to the MPR 300 column this fraction may be as low as a few per cent. In order to reduce dephosphorylation during the processing
- 30 steps the presence of one or more phosphatase inhibitors appears crucial. Such phosphatase inhibitors are available from commercial sources.

An important aspect of the invention relates to a recombinant alpha-mannosidase which is obtainable and/or obtained by the method of the invention and to the use of this enzyme

- 35 for the preparation of a medicament for the treatment of alpha-mannosidosis. In particular, it is preferred that the alpha-mannosidase is a recombinant human alpha-mannosidase.

WO 2005/094874

PCT/DK2005/000228

28

The disease, which is the target for the inventive method is alpha-mannosidosis, and therefore the catalyst is alpha-mannosidase or an enzymatically equivalent part or analogue thereof. It is most preferred that the catalyst is a recombinant form of the human alpha-mannosidase enzyme or of the enzymatically equivalent part or analogue thereof, since recombinant production will allow large-scale production which, with the present means available, does not seem feasible if the enzyme would have to be purified from a native source.

Preferably, the alpha-mannosidosis is made by recombinant techniques. In a further embodiment, the alpha-mannosidosis is human (hLAMAN) and still more preferred mature human alpha-mannosidase (mhLAMAN) or a fragment thereof. The fragment may be modified, however the active sites of the enzyme should be preserved.

It is to be expected that, in preparations of alpha-mannosidosis according to the present invention, one fraction of the enzyme is represented by its precursor form, while other fractions represent the proteolytically processed forms of approximately 55 and 70 kDa.

In general, the target cell is a cell wherein the enzymatic activity, such as the alpha-mannosidase activity is insufficient for the optimal function of the cell. Insufficient activity of alpha-mannosidase may be measured by one or more of the parameters selected from monitoring increased levels of mannose-rich oligosaccharides in urine, analysis of alpha-mannosidase activity in material from a subject such as in leukocytes and/or in skin fibroblasts, presence of clinical symptoms or increase in rate of development of clinical symptoms of alpha-mannosidosis.

A significant feature of insufficient alpha-mannosidase activity is a cell wherein a massive intracellular accumulation of mannose-rich oligosaccharides is present. Naturally, such cell is a target cell according to the present invention. The target cell may also be a cell of the nervous system. In addition, target cells for delivering the enzyme also includes one or more cell types selected from human monocytes, human fibroblasts, human lymphocytes, human macrophages.

An increased activity of the alpha-mannosidase may be used as a parameter for evaluating the success of a treatment schedule. The activity be measured by one or more of the parameters selected from monitoring increased levels of mannose-rich oligosaccharides in urine, analysis of alpha-mannosidase activity in material from the patient such as in leukocytes and/or in skin fibroblasts, presence of clinical symptoms or increase in rate of development of clinical symptoms of alpha-mannosidosis.

WO 2005/094874

PCT/DK2005/000228

29

It is a very important aspect of the invention to perform the treatment of a possible enzyme defect prenatally. In a further aspect of the invention, the cellular membrane is the fetal-maternal barrier (placenta). As is the case for the blood brain barrier it may be an important characteristic of the present invention that actual delivery of the enzyme over  
5 the fetal-maternal barrier or disruption of the fetal-maternal barrier is unnecessary and undesired.

With respect to the above description of the various aspects of the present invention and of the specific embodiments of these aspects it should be understood that any feature and  
10 characteristic described or mentioned above in connection with one aspect and/or one embodiment of an aspect of the invention also apply by analogy to any or all other aspects and/or embodiments of the invention described.

When, in the present application, an object according to the present invention or one of its  
15 features or characteristics is referred to in singular this also refers to the object or its features or characteristics in plural. As an example, when referring to "a cell" it is to be understood as referring to one or more cells.

Throughout the present specification the word "comprise", or variations such as  
20 "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

## 25 LEGENDS TO FIGURES

Fig. 1: Polypeptide pattern of bovine, mouse and human LAMAN

10  $\mu$ g of LAMAN purified from bovine kidney or the secretions of cell overexpressing mouse or human LAMAN were separated by SDS-PAGE and stained with Coomassie Blue. The 130  
30 kDa polypeptides seen in mouse and human LAMAN correspond to the precursors and the polypeptides ranging from 11 to 70 kDa to proteolytically processed forms of LAMAN. The 46-48 kDa polypeptide in bovine LAMAN represents partially processed intermediates.

Fig. 2: Thin-layer chromatography of neutral oligosaccharides in liver, spleen, kidney and  
35 heart.

WO 2005/094874

PCT/DK2005/000228

30

The fraction of neutral oligosaccharides from equal amounts of tissues was separated by thin-layer chromatography. Lane 1 contains the sample from control mice, lane 2 from mock-injected LAMAN mice, lane 3, 4 and 5 the samples from  $\alpha$ -mannosidosis mice injected with 100 mU mouse LAMAN per g body weight and killed after 2, 4 and 10 h, respectively (see also Table 1). Oligosaccharides were detected with orcinol/ sulphuric acid. Mass spectrometry and sensitivity to jack bean  $\alpha$ -mannosidase demonstrated that the oligosaccharides M2 to M9 contain 2 to 9 mannose residues and a single N-acetylglucosamine residue at the reducing terminus. The oligosaccharides were quantified by densitometry. For calculation of the storage the values for neutral oligosaccharides in  $\alpha$ -mannosidosis mice were corrected for the neutral oligosaccharides in control mice and expressed as percentage of that in mock-injected  $\alpha$ -mannosidosis mice. The storage in LAMAN injected  $\alpha$ -mannosidosis mice was expressed as percentage of that in mock-injected  $\alpha$ -mannosidosis mice. Mass spectrometry and sensitivity to jack bean  $\alpha$ -mannosidase showed that the oligosaccharides M2 to M9 contain 2 to 9mannose residues and a single Nacetylglucosamine residue at the reducing terminus.

Fig. 3: Neutral oligosaccharides in tissue extracts of  $\alpha$ -mannosidosis mice injected with 100 mU mouse LAMAN per g body weight.

The mice were killed 4, 8 and 16 days after injection and the neutral oligosaccharides in extracts of liver, spleen, kidney and heart were quantified by densitometry after separation by thin layer chromatography (see legend of Fig. 2).

Fig. 4: Clearance of LAMAN from serum

LAMAN of bovine ( $\square$ ), mouse ( $\blacksquare$ ) or human (O) origin, 50 mU/g body weight, was injected into the tail vein of 8 weeks old  $\alpha$ -mannosidosis mice. Blood was collected from the retroorbital plexus 5 to 65 min after injection. Each value represents the mean of two animals. The range is indicated by bars where it exceeds the size of the symbols.

Fig. 5: Corrective effect of bovine, mouse and human LAMAN on the storage of neutral oligosaccharides

Mice received 50 mU LAMAN per g body weight (see Fig. 3) 2 days prior tissue analysis. Neutral oligosaccharides were quantified as in Figs. 2 and 3. The bars indicate the range observed in two independent mice.



WO 2005/094874

PCT/DK2005/000228

31

Fig. 6: Neutral oligosaccharides in tissue extracts of  $\alpha$ -mannosidosis mice after injection of a single dose of 250 mU of human  $\alpha$ -mannosidase per g body weight

The mice were killed 1, 3, 6 and 12 days after injection. The neutral oligosaccharides in the tissue extracts of liver, spleen, kidney and heart were quantified by thin-layer

5 chromatography and densitometry as in Figs. 1 and 2.

Fig. 7: Disappearance and reappearance of storage vacuoles in liver

A, C, E, light microscopy (semithin sections); B, D, F, ultrastructure of Kupffer cells; A, B, mock-injected  $\alpha$ -mannosidosis mouse (age 14 weeks). Hepatocytes show clear vacuoles  
 10 along the bile canaliculi (A, arrows). The heavily vacuolated sinus wall cells are not clearly discerned at the light microscopic level (A) because of the extremely narrow cytoplasm bridges between the vacuoles as seen in (B). C, D,  $\alpha$ -mannosidosis mouse, 1 day after injection of 250 mU human LAMAN per g body weight. Vacuoles have disappeared from hepatocytes (C). Very few vacuolated sinus wall cells are seen (C, arrow). The vacuoles in  
 15 Kupffer cells are smaller than in the mock-treated animal and can contain an electron-dense matrix (D). E, F,  $\alpha$ -mannosidosis mouse, 12 days after injection of 250 mU human LAMAN per g body weight. Vacuoles have reappeared in sinus wall cells (E, arrows) and less so in hepatocytes. In Kupffer cells (F) the vacuoles qualitatively resemble those in mock-injected mice. The dense inclusions in the hepatocytes shown in A, C, and E  
 20 represent lipid droplets, which are equally encountered in the corresponding wild type mice (not shown). Bars represent 20  $\mu$ m and 2  $\mu$ m, respectively.

Fig. 8: Neutral oligosaccharides in tissue extracts of  $\alpha$ -mannosidosis mice twice injected with 250 mU human LAMAN per g body weight

25 Control (lane 1) and  $\alpha$ -mannosidosis mice (lane 2 and 3) were injected with PBS (lane 1 and 2) or 250 mU human LAMAN per g body weight (lane 3) 7 and 3.5 days prior analysis. Neutral oligosaccharides were prepared and quantified as in Fig. 1 and 2. The migration of oligosaccharides composed of 2 to 9 mannose residues and a single N-acetylglucosamine residue (M2 to M9) is indicated. Arrows mark orcinol-positive material insensitive to  
 30 LAMAN. The results in a duplicate set of mice (not shown) varied by less than 15%.

Fig. 9: Light microscopy of kidney in  $\alpha$ -mannosidosis mice

$\alpha$ -Mannosidosis mice were injected with PBS (A) or 250 mU human LAMAN per g body weight (B) 7 and 3.5 days prior killing. The outer stripe of the outer medulla is shown.

WO 2005/094874

PCT/DK2005/000228

32

Numerous clear vacuoles are seen in the thick ascending limb (TAL) of Henle's loop of the mock-injected mouse (A, arrows) in contrast to the LAMAN-treated mouse (B). The proximal straight tubules (PST) are free of pathological vacuoles in either animal. Bars represent 20  $\mu\text{m}$ .

5

Fig. 10: HPLC-separation of 2-anthranilamide derivatized neutral oligosaccharides from brain

Neutral oligosaccharides from brain of  $\alpha$ -mannosidosis mice injected with PBS (upper scan) or 250 mU human LAMAN per g body weight (lower scan) 7 and 3.5 days prior killing were  
10 mixed with 220 pmol  $\text{GlcNAc}_4\text{Man}_3\text{Gal}_3$  as an internal standard, derivatized with 2-anthranilamide and separated by HPLC. The position of the Internal standard and of oligosaccharides composed of 2 to 9 mannose residues and a single N-acetylglucosamine residue (M2 to M9) are Indicated.

## 15 EXAMPLES

### Example 1: Production and characterisation of LAMAN

#### Experimental procedures

#### 20 *Expression and purification of recombinant human LAMAN in CHO cells*

Human LAMAN cDNA isolated from HepG2 cDNA library and subcloned into an expression vector carrying a dihydrofolate reductase gene and the LAMAN cDNA under the control of the human CMV-promotor was expressed in Chinese Hamster Ovary (CHO) cells deficient in dihydrofolate reductase. The CHO cells were cultured in a two-compartment CELLline  
25 flask (Integra Biosciences Inc.) in serum free ExCell 302 medium (JRH Biosciences) supplemented with 20 nM methotrexate at 37°C in a humidified atmosphere containing 5%  $\text{CO}_2$ . The medium was diafiltrated using a Pellicon Biomax polysulphone filter with a 100 kDa cut off against 4 volumes 0.02 M Tris-HCl, pH 7.6. Ion-exchange chromatography was performed on DEAE-Sepharose FF (Amersham Pharmacia Biotech AB) using a NaCl  
30 gradient in 0.02 M Tris-HCl, pH 7.6. Active fractions were concentrated using an Amicon Centricon Plus-80 centrifugation filter with a 30 kDa cut off and subjected to gel filtration in a HiPrep 26/60 Sephacryl High Resolution column (Amersham Pharmacia Biotech AB) in

WO 2005/094874

PCT/DK2005/000228

33

0.02 M Tris-HCl, pH 7.6, containing 0.15 M NaCl. After concentration the final preparation had a specific activity of 9-15 U/mg.

*Expression and purification of recombinant mouse LAMAN*

- 5 The cDNA encoding the mouse LAMAN (16) was subcloned in the expression vector pMPSVEH (17). A polyhistidine tail (6 residues) had been added to the C-terminus part of the enzyme. Mouse embryonic fibroblasts deficient in the small and the large mannose 6-phosphate receptors (mpr<sup>-/-</sup> MEF) (18) were transfected and stably expressing clones were selected with 50 µl/ml hygromycin. For production of recombinant mouse LAMAN the
- 10 cells were cultured in medium supplemented with 10% FCS in a humidified atmosphere containing 5% CO<sub>2</sub>. The secreted recombinant mouse LAMAN was purified from conditioned medium using a three step procedure. In the first step the medium was dialyzed against 20 mM sodium phosphate buffer pH 7,8 containing 500 mM sodium chloride and then loaded onto a Probond column (Invitrogen). The retained enzyme was eluted with a
- 15 gradient 0 to 0.35 M of imidazole (total volume 80 ml) in 20 mM sodium phosphate buffer pH 6.0 containing 500 mM sodium chloride. The LAMAN containing fractions were dialyzed against 10 mM sodium phosphate, pH 6.0 and loaded onto a DEAE-cellulose. The enzyme was eluted in a 0 to 0.25 M sodium chloride gradient (total volume 80 ml) in 10 mM sodium phosphate buffer. Finally mouse LAMAN was adsorbed to ConA-Sepharose (loading
- 20 buffer 20mM Tris-HCl, pH 7.4, containing 1 mM MgCl<sub>2</sub>, 1mM MnCl<sub>2</sub>, 1 mM CaCl<sub>2</sub> and 0.5 M NaCl), and eluted with α-mannopyranoside (0.0-1.0 M) in the same buffer. The final preparation had a specific activity of 17-25 U/mg.

*Purification of bovine LAMAN*

- 25 Bovine LAMAN was purified from kidney as described (19). The final preparation had a specific activity of 10 U/mg.

*Purification of human LAMAN*

- LAMAN was expressed in Chinese Hamster Ovary (CHO-) cells that was cultured in a two
- 30 compartment CellLine flask, at 37°C in a humidified atmosphere with 5 % CO<sub>2</sub>. The enzyme, obtained from the CHO-cells, was purified according to a purification procedure consisting of 4 steps:

*Diafiltration*

- The diafiltration was based on the TFF model, using a Pellicon Biomax polysulphone filter
- 35 with a 100 kDa-cut off. The sample was diafiltrated against approximately 3 × sample volume with 0.02 M Tris-HCl, pH 7.6.

WO 2005/094874

PCT/DK2005/000228

34

### Ion exchange chromatography

Ion exchange chromatography was performed using DEAE Sepharose flow filtration. Buffer was 0.02 M Tris-HCl pH 7.6. The gel was packed in a XK 16/20 column from Amersham Pharmacia Biotech AB. The elution was performed with a gradient of NaCl.

5

### Concentration of sample

Prior to Gel filtration, the sample was concentrated to a protein concentration of ~6 mg/mL and a final volume of 10 mL. This was carried out using a Amicon Centricon Plus-80 centrifugation filter with a 30 kDa cut off.

10

### Gel filtration

Gel filtration was carried out in a HiPrep 26/60 Sephacryl High Resolution column from Amersham Pharmacia Biotech AB. The sample was eluted with 0.02 M Tris-HCl supplied with 0.15 M NaCl, pH 7.6.

15

Example of a LAMAN purification is summarized in the table below. Normally Yield is in the range of 30 – 50 % and specific activity 9 – 15 U/mg.

#### *Summary of the LAMAN purification*

| Purification step | Protein concentration (mg/mL) | Specific activity (U/mg) | Purification factor | Yield (%) |
|-------------------|-------------------------------|--------------------------|---------------------|-----------|
| Crude enzyme      | 3.3                           | 0.89                     | 1.00                | 100       |
| Diafiltration     | 2.0                           | 2.28                     | 2.56                | 130       |
| Ion exchange      | 1.4                           | 3.61                     | 4.06                | 54        |
| Concentration     | 5.8                           | 6.01                     | 6.75                | 66        |
| Gel filtration    | 0.8                           | 10.87                    | 12.21               | 64        |
| Concentration     | 6.4                           | 9.65                     | 10.84               | 32        |

20 The purified LAMAN is stored at -80 °C.

In addition, a purification process for rhLAMAN in 20-200 ml scale is developed for scale-up to large scale production. The quality and purity of the final product (rhLAMAN) is very

WO 2005/094874

PCT/DK2005/000228

35

high and according to the specifications (approved for clinical trials). The process includes a capture step, 1-2 intermediate purification steps, 1 polishing step, 1-2 virus removal steps and 1 formulation step. 1 or more buffer exchange steps are also included (dia-filtration). The small-scale process is transferred to intermediate and finally large-scale  
5 production.

Experimental design: Several different chromatography gels are tested and performance of the different steps will be analysed by a battery of analytical methods described briefly below:

10

|                                     |                           |
|-------------------------------------|---------------------------|
| <u>Enzyme activity:</u>             | Alpha-mannosidase assay   |
| <u>Total protein concentration:</u> | BCA analysis              |
| <u>rhLAMAN concentration:</u>       | rhLAMAN ELISA             |
| <u>Purity:</u>                      | HPLC and SDS-PAGE         |
| 15 <u>Identity:</u>                 | HPLC                      |
| <u>HCP proteins:</u>                | ELISA                     |
| <u>Endotoxin level:</u>             | Outsource to contract Lab |

Outline of purification process in 20 ml column scale

20

Step 1: Concentration/Diafiltration

Media produced in T-500 flasks (0.06U/ml) with expressed rhLAMAN is concentrated approximately 10x for using Tangential flow filtration (TFF) against a Pellicon Biomax polysulphone filter with 100 kDa cut off.

25

Example: 0.5 – 1.0 litre spinner produced media is concentrated using TFF against a 50 cm<sup>2</sup> Biomax 100 kDa filter to 50 - 100 ml. Transmembrane pressure (TMP) is 25 (Pin = 30 psi; Pout = 20 psi).

After that, diafiltration against 7 volumes of 20 mM Tris-HCl, pH 7.6 with TMP of 25 is

30 applied. Specific activity of concentrated sample is 0.5 – 1.5 U/mg. Yield 70 – 90%

Step 2: Capture step – DEAE sepharose FF

Concentrated sample from step 1 is applied on a 20 ml DEAE sepharose packed in a 16 mm diameter column (Pharmacia XK 16) equilibrated with 20 mM Tris-HCl pH 7.6 (referred

35 to as: standard buffer). Flow rate is 3 ml/min. Protein bound to the DEAE gel is then

WO 2005/094874

PCT/DK2005/000228

36

washed with 2-4 column volumes (CV) of standard buffer followed by 2-4 CV's of 30 mM NaCl in standard buffer.

rhLAMAN is eluted with a linear gradient from 30 – 300 mM NaCl in standard buffer for 20  
5 minutes. Alternatively for the large scale production: Elution is achieved by applying 75 –  
150 mM NaCl in standard buffer. The DEAE gel is washed with 2-4 CV's of 0.5 M NaCl in  
standard buffer followed by 2.4 CV's of 1.0 M NaOH (contact time 40 – 60 minutes).  
Fractions containing rhLAMAN activity are pooled (specific activity: 2 – 5 U/mg) and used  
for further purification. Yield 70 – 90 %.

10

#### Step 3: Intermediate step 1

1. *Hydrophobic interaction chromatography: butyl, phenyl or octyl sepharose FF.*

Sample pool from step 2 is mixed 1:1 with 2.0 M Na<sub>2</sub>SO<sub>4</sub> and applied on a 20 ml  
15 HIC column (butyl-, phenyl- or octyl-sepharose) packed in a 16 mm diameter  
column (Pharmacia XK 16) equilibrated with standard buffer + 1.0 M Na<sub>2</sub>SO<sub>4</sub>. Flow  
rate is 3-4 ml/min. Column is washed with 2-4 CV's of the same buffer. rhLAMAN  
is eluted with standard buffer and fractions containing activity are pooled and used  
for further purification.

20

2. *Macrorep, Ceramic hydroxyapatite type I or II.*

*Brief description:* Equilibrate column (Gel volume = 20 ml) with 4-6 CV's of 10 mM  
Sodium phosphate H 7.6 (Buffer A). Flow rate 2-5 ml/min. Buffer exchange sample  
from step 3 with TFF to Buffer A. Load sample from step 3 on column. Wash with  
25 2-4 CV's of Buffer A. Elute with buffer A containing 100 mM NaCl. Collect peak  
containing rhLAMAN activity.

#### Step 4: Intermediate step 2

- 30 1. *Macro prep, Ceramic hydroxyapatite type I or II, 40 um.*

*Brief description:* see Step 3

2. *Source 15 Q – anioexchanger*

Equilibrate column with 4 CV's of 200 mM Tris-HCl pH 7.6. Change to 5 CV's of 20  
35 mM Tris-HCl pH 7.6 (standard buffer). Flow rate: 2- 5 ml/min. Load sample from  
step 3 (should be in standard buffer before application) onto column. Wash with 2-  
4 CV's of standard buffer. Apply a shallow gradient from 0 to 100% of 1 M NaCl in  
standard buffer (flow rate 2ml/min, gradient time 50 minutes). Collect fractions  
containing rhLAMAN activity.

WO 2005/094874

PCT/DK2005/000228

37

3. *Source 15 S – cation exchanger*

Should run in acidic pH. Equilibration buffer = 20 mM Sodium Acetate pH 4.5. Elute with a NaCl (increasing salt concentration) or pH (increasing pH) gradient.

5

Step 5: Polishing step

1. *Macro prep, Ceramic hydroxyapatite type II, 40 um.*

Description of parameters: see Step 3.

10

2. *Source 15 Q – anion exchanger*

Description of parameters: see Step 4.

3. *Source 15 S – cation exchanger*

15 Description of parameters: see Step 4.

4. *Affinity chromatography*

20 Step 6: Diafiltration / Formulation step

Tangential flow filtration (TFF) against a Pellicon Biomax polysulphone filter with 100 kDa cut off against 5-10 x Volumes of formulation buffer is performed. Two formulation buffers are tested:

25 Formulation buffer 1.

|                                  |              |
|----------------------------------|--------------|
| Na <sub>2</sub> HPO <sub>4</sub> | 3.10-3.50 mM |
| NaH <sub>2</sub> PO <sub>4</sub> | 0.4-0.6 mM   |
| Glycine                          | 25-30 mM     |
| Mannitol                         | 220-250 mM   |

30 Water for injection (WFI)

Formulation buffer 2.

|                |            |
|----------------|------------|
| Sodium Citrate | 4.0-5.0 mM |
| Citric Acid    | 0.3-0.8 mM |

35 Mannitol 200-250 mM

|          |            |
|----------|------------|
| Tween 80 | 3.0-5.0 mM |
|----------|------------|

Water for Injection (WFI)

WO 2005/094874

PCT/DK2005/000228

38

The pH and osmolality in both Formulation buffers are balanced to  $7.5 \pm 0.2$  and  $300 \pm 50$  mOsm/kg respectively. Final protein concentration is according to the specification ( $>5$  mg/ml).

5 Step 7: Formulation, Filling and Freeze-drying

*Formulation and dosage form*

In the development of dosage form the stability of rhLAMAN is focused. The development process starts with an aqueous solution and will, most likely, end up as a freeze-dried product. Two different formulations are tested: Formulation buffer 1 and Formulation

10 buffer 6, see Step 6.

Both these formulations are known to stabilize proteins in aqueous solutions as well as in freeze-dried powders. The pH and osmolality in both Formulation buffers will be balanced to  $7.5 \pm 0.2$  and  $300 \pm 50$  mOsm/kg respectively. Final protein concentration should be

15 according to the specification and in the range 4-10 mg/ml.

A freeze-dried product of rhLAMAN will be produced at a production unit according to EU GMP practice. The filling and freeze-drying will be performed in a room classified as Class A. During production the filling zone is monitored with particle count and settle plates. The

20 personnel are regularly trained according to EU GMP and monitored after each production with glove prints. The sterility of equipment and materials are secured by validated sterilization procedures.

*Filling*

25 The bulk drug substance of rhLAMAN are aseptically filled in sterile type I glass vials.

*Freeze-drying*

The vials are freeze-dried with freeze-drying cycles specifically developed for rhLAMAN in the two different formulations described above. Nitrogen gas is filled into the vials in the

30 end of the cycle and eventually closed with stoppers and capped. The batch is finally analyzed and released according to the specification.



WO 2005/094874

PCT/DK2005/000228

39

*Determination of phosphorylation of LAMAN*

LAMAN was incubated overnight with a MPR 300 affinity matrix as described (20). LAMAN activity was determined in the unbound fraction and the fractions eluted with 5 mM glucose 6-phosphate and 5 mM mannose 6-phosphate.

5

## Results

- LAMAN purified from three different species was used for enzyme replacement in  $\alpha$ -mannosidosis mice (Fig. 1). The LAMAN from bovine kidney is represented by a mixture of
- 10 polypeptides (11-38 kDa) generated by limited proteolysis from a common precursor (19). As expected for a lysosomal hydrolase purified from tissue and thereby having been exposed to endosomal/ lysosomal phosphatase activity, the enzyme has a low content of Man6P-recognition marker. When incubated with a Man6P-receptor affinity matrix, 6.4% of the LAMAN activity bound to the matrix in a Man6P-dependent manner.
- 15 The recombinant mouse and human LAMAN were isolated from the secretions of Man6P-receptor deficient mouse fibroblasts and of CHO cells, respectively. The enzymes were largely represented by their precursor forms, but contained a variable fraction (5-35%) of proteolytically processed forms of 55 and 70 kDa (Fig. 1). The mouse LAMAN had a higher content of Man6P-recognition marker, which mediated binding of 73.6% of the activity to
- 20 the affinity matrix. Of the human LAMAN only 4.2% bound in a Man6P-dependent manner to the Man6P-receptor affinity matrix, indicating that the content of Man6P-recognition marker is as low as for the bovine LAMAN.

Several batches have subsequently been analysed, for example:

- 25 pooled batch 2003-09 - 2004-02: 40 % phosphorylation  
batch 2004-02-02 - 06: 30 % phosphorylation  
pooled batch 2003-01-21 - 04-16 42 % phosphorylation

- In general approximately 40% of the amount of enzyme binds to the affinity matrix. It is
- 30 believed that the low degree of phosphorylation observed for the human and bovine LAMAN results from prolonged storage of the enzymes.

Example 2: Effects of the in vivo administration of LAMAN on the levels of stored oligosaccharides

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WO 2005/094874

PCT/DK2005/000228

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## Experimental procedures

### *Injection of the mice*

LAMAN was injected into the tail vein of 8-14 week old  $\alpha$ -mannosidosis mice (final volume up to 5,3  $\mu$ l/g body weight). Mock-injected mice received the same volume of 10 mM phosphate, pH 7.4 in 0.15 M NaCl (PBS). In a single experiment the mice originating from up to three litters did not differ in age. 5 min after injection, blood was taken from the retroorbital plexus to control for the amount of injected enzyme. Serum was prepared and stored at -20°C.

### 10 *Preparation of organ extracts*

Mice were anaesthetized with 20  $\mu$ l of a solution of 10 mg/ml Ketavet (Parke Davis) and 2 mg/ml Rompun (Bayer) in 0.15 M NaCl and perfused with PBS. Organs (liver, spleen, kidney, heart and brain) was collected and stored at -78°C. About 50-70 mg of each organ were homogenized at 4°C in 9 volumes (per weight) of 10 mM Tris/HCl, 150 mM NaCl, 1 mM PMSF (in isopropanol), 1 mM iodoacetamide, 5 mM EDTA. Triton X-100 was added to a final concentration of 0.5% w/v (1% for liver). After incubation for 30 minutes on ice, the samples were sonicated and then centrifuged for 15 minutes at 13000 g. The supernatant was stored at -20°C.

### *Enzyme assays*

20 For determination of LAMAN activity in the organ extracts and in serum, 10-50  $\mu$ l of enzyme sample, was incubated in 0.2 ml of 0.2 M sodium citrate pH 4.6, 0.08% NaN<sub>3</sub>, 0.4% BSA, 0.15% NaCl, and 10 mM p-nitrophenyl- $\alpha$ -mannopyranoside as substrate for 0.5-5h at 37°C. 1 ml of 0.4 M glycine/NaOH, pH10.4 was added to stop the reaction. Absorbance was read at 405 nm ( $\epsilon = 18500 \text{ M}^{-1}\text{cm}^{-1}$ ). All the determinations were done in duplicate and with the appropriate blanks.

### *Western blotting*

For Western blotting of human LAMAN 20-40  $\mu$ g of protein was separated on a 10% SDS-polyacrylamide gel. After SDS-PAGE electrophoresis, the proteins were transferred to PVDF membranes using a semi-dry blotting system. Transfer efficiency was checked with Ponceau staining. The membranes were subsequently blocked with 5% skimmed milk powder and incubated with the primary antibody in a 1:50.000 or 1:100.000 dilution. After washing in PBS, 0.1% Tween 20, the blots were incubated with horseradish peroxidase (HRP) coupled secondary antibodies (1:20.000). Signals were visualised using the ECL-Detection System (Amersham, Freiburg, Germany).

WO 2005/094874

PCT/DK2005/000228

41

*Isolation of neutral oligosaccharides*

Tissue samples (50-60 mg) were cut into small pieces and homogenized with 0.6 ml H<sub>2</sub>O (HPLC grade) at 4°C. After freezing (-20°C) and thawing twice and ultrasonic treatment, proteins were precipitated by the addition of 4 vol methanol and extracted by the addition  
5 of 4 vol chloroform/H<sub>2</sub>O (1:3) (21). The supernatant was desalted by incubation for 1 h at 4°C with mixed-bed ion-exchange resin (AG 501-X8, 20-50 mesh). The unbound material was lyophilized and resuspended in water (1 ml per mg tissue).

*Separation of mannose oligosaccharides by thin layer chromatography (TLC)*

Neutral oligosaccharides extracted from equal aliquots of tissue were loaded onto TLC  
10 plates (20x20 Silica gel F60, Merck). After drying the plates at room temperature for ~ 1h, the oligosaccharides were separated by developing overnight with n-butanol / acetic acid / H<sub>2</sub>O (100:50:50). After drying (~ 1h at room temperature and then 5 min at 110°C), the plates were developed for 4 h in n-propanol / nitromethan / H<sub>2</sub>O (100:80:60). For staining, the plates were sprayed with 0,2% orcinol in H<sub>2</sub>SO<sub>4</sub> (20% in water), and heated at 110°C.  
15 The size of the oligosaccharides was determined by MALDI-TOF (see below).

*Digestion of liver oligosaccharides with  $\alpha$ -glucosidase or jack bean  $\alpha$ -mannosidase*

To avoid the interference with glycogen derived oligosaccharides 20  $\mu$ l of the oligosaccharide extracts from liver and heart were incubated overnight at 37°C with 40 U/ml  $\alpha$ -glucosidase from *Bacillus stearothermophilus* (Sigma) in 20 mM phosphate, pH 6.8.  
20 The incubation mixture was heated at 96°C to denature the proteins. After centrifugation 10 min at 13000 g, the supernatant was desalted by incubation 1h at 4°C with a ion-exchange resin (AG 501-X8, 20-50 mesh), lyophilized and resuspended in 20  $\mu$ l of water, and then separated by TLC.

To verify the nature of the oligosaccharides, 20  $\mu$ l of the oligosaccharide extracts were  
25 incubated overnight at 37°C with 30 U/ml  $\alpha$ -mannosidase from Jack bean (Sigma) in 0.1 M sodium acetate, pH 5.0, containing 2 mM ZnCl<sub>2</sub>. After incubation the oligosaccharides were prepared as described for samples digested with  $\alpha$ -glucosidase and separated by TLC.

*Quantitative analysis of neutral oligosaccharides in organs*

The oligosaccharides (0.3  $\mu$ l) were mixed with 220 pmol of a decasaccharide which served  
30 as an internal standard and had the composition GlcNAc<sub>4</sub>Man<sub>3</sub>Gal<sub>3</sub>. The mixture was lyophilized and resuspended in 5  $\mu$ l of a DMSO / acetic acid (7:3) containing 0.34 M 2-anthranilamide (Aldrich) and 1 M NaBH<sub>3</sub>CN (Fluka). After incubation 2 h at 65°C, the samples were purified by paper chromatography (developed with ethyl acetate). The oligosaccharides, localized at the starting point, were extracted by sonicating the paper in  
35 water, lyophilized, resuspended in 300  $\mu$ l acetonitrile / 80 mM ammonium formiate pH 4.4 (65:35), loaded onto a Gluco-Sepharose column (Ludger) and eluted at a flow rate of 0.4

WO 2005/094874

PCT/DK2005/000228

42

ml/min with the acetonitrile / ammonium formate buffer. Fluorescence (excitation 350 nm, emission 450 nm) was recorded (Shimadzu, RF-10A XL) and the mass of the oligosaccharides determined by MALDI-TOF.

#### *MALDI-TOF*

- 5 Samples from HPLC fractions were lyophilised and dissolved in 2-3  $\mu$ l water. Water extracts from TLC plates were derivatized with 1-phenyl-3-methyl-5-pyrazolone and dissolved in 2-3  $\mu$ l water. 2,5-dihydroxybenzoic acid (DHB, 5 mg/ml in water) was used as matrix. 0.5  $\mu$ l of DHB and 1  $\mu$ l of sample were spotted onto the Anchorchip target (Bruker Daltonik), and dried under room temperature. Mass spectrometric analysis was performed
- 10 on a Reflex III MALDI-TOF (Bruker Daltonik) with a 337nm UV laser.

#### *Histological examinations*

- For electron microscopy, small sections of liver and kidney collected at killing time were immersed in 0.1 M phosphate, 6% glutardialdehyde pH 7.4. Tissue samples were post-fixed with 2% osmium tetroxide, dehydrated and embedded in Araldite. Semi-thin sections
- 15 were stained with toluidine blue. Ultrathin sections were processed according to standard techniques. For histochemical investigations, the sections were immersed with Bouin's solution diluted 1:4 in 10 mM phosphate pH 7.4, 0.15 M NaCl. Embedding was performed in low melting point paraffin (Wolff, Wetzlar, Germany). Serial sections (7  $\mu$ m) were cut and mounted on glass slides covered with Biobond (British Biocell, London, UK). Central
- 20 sections of each series were stained with haematoxylin and eosin for standard light microscopy.

#### **Results**

##### **Corrective effect of a single intravenous injection of mouse LAMAN**

- 25 In order to study the short and the long term effect of a single dose of LAMAN on the storage of neutral oligosaccharides,  $\alpha$ -mannosidosis mice at the age of 9 weeks received 100 mU mouse LAMAN per g body weight intravenously. To control for the amount of injected enzyme and its clearance from circulation blood was taken 5, 30 and 60 min after injection. 5 min after injection the LAMAN activity varied by less than  $\pm$  6% indicating that
- 30 the mice had received comparable amounts of enzyme. The enzyme was cleared from circulation with a half life of less than 20 min. The mice were killed 2, 4 and 10 h after injection and organ extracts were prepared for determination of LAMAN activity and neutral oligosaccharides. The maximum activity of LAMAN in the organs was observed 2-4 h after injection (Table 1). In liver the activity was 6-7 times higher than in control mice,
- 35 but also in spleen and heart the maximum values exceeded those of controls. In kidney at

WO 2005/094874

PCT/DK2005/000228

43

maximum one fourth of the activity in control mice was reached. The small activity seen in brain is likely to be LAMAN present in the vascular system. Between 4 and 10 h after injection the enzyme activity decreased rapidly in liver, spleen and kidney with an apparent half life of about 3h. Western blot demonstrated that the internalised precursor of

5 LAMAN was rapidly processed to mature forms (not shown).

The hallmark of  $\alpha$ -mannosidosis is the storage of neutral oligosaccharides in a wide variety of tissues (see Fig. 2, lane 1 and lane 2). These oligosaccharides contain 2-9 mannose residues and an N-acetylglucosamine residue at their reducing terminus and therefore result from the action of an endoglucosaminidase. The amount of neutral oligosaccharides

10 in liver and spleen decreased progressively with time to 15% and to 7% of that in mock-injected animals, while in kidney and heart the storage was reduced only to about 49% and 74% (Fig. 2).

| Genotype      | LAMAN injected (100mU/g body weight) | LAMAN (mU/g wet weight) |                   |                   |                 |                |
|---------------|--------------------------------------|-------------------------|-------------------|-------------------|-----------------|----------------|
|               |                                      | Liver                   | Spleen            | Kidney            | Heart           | Brain          |
| (+/+)*<br>(n) | -                                    | 136,7±29,1<br>(19)      | 116,3±44,5<br>(3) | 168,4±34,5<br>(9) | 12,6±3,1<br>(3) | 25±1<br>(2)    |
| (-/-)*<br>(n) | -                                    | 2,9±2,5<br>(21)         | 2,8±2,1<br>(4)    | 2,5±1,1<br>(10)   | 0,6±0,5<br>(3)  | 0,4±0,3<br>(3) |
| (/-/-)*       | 2h <sup>+</sup>                      | 1057                    | 185               | 26                | 22              | 2,9            |
|               | 4h <sup>+</sup>                      | 883                     | 183               | 40                | 5               | 1,4            |
|               | 10h <sup>+</sup>                     | 197                     | 36                | 10                | 2               | 1,0            |

15 **TABLE 1:** LAMAN activity in tissue extracts of control and  $\alpha$ -mannosidosis mice before and after injection of 100 mU mouse LAMAN per g body weight. \* +/+ refers to control mice, -/- to  $\alpha$ -mannosidosis mice, and n to the number of animals investigated.<sup>+</sup> All values were corrected for the mean  $\alpha$ -mannosidase activity in serum at  $t_{zero}$  (2350 mU/ml serum). The correction factors were 0,98, 1,07 and 0,96 for 2, 4 and 10h respectively.

20

To determine how long the corrective effect of a single dose of LAMAN persists, mice were examined 4, 8 and 16 days after the injection of 100 mU mouse LAMAN per g body weight.

WO 2005/094874

PCT/DK2005/000228

44

At all time points the LAMAN activity in the organs (spleen, kidney, heart, brain) was in the range of non-treated or mock-injected  $\alpha$ -mannosidosis mice, except for liver, where 4 days after injection the LAMAN activity (15.1 mU/g wet weight) was still about 5-fold higher than in mock-injected mice. The storage of neutral oligosaccharides in liver, spleen and kidney 4 days after injection was in the range seen 10 h after injection (compare Fig. 2 and Fig. 3). In heart storage had decreased from 74% after 10 h to 42% after 4 days. This indicates that the corrective effect seen after 10 h persists for about 4 days in spite of the fact that little or no LAMAN activity is detectable 10 h after injection in organs such as kidney or heart. After 4 days the storage of oligosaccharides clearly began to increase again. The increase observed between day 4 and day 16 after injection corresponded to 20-40% of the storage seen in mock-injected  $\alpha$ -mannosidosis mice (Fig. 3).

Comparison of the clearance and corrective effect of bovine, mouse and human LAMAN

To compare the corrective effect of the LAMAN preparations from the three sources mice were injected with a dose of LAMAN that was expected to yield a partial correction. We therefore injected 50 mU LAMAN per g body weight. The bovine and the human LAMAN were rapidly cleared from circulation with half lifes of 4 min and 8 min, respectively (Fig. 4). The clearance of the highly phosphorylated mouse LAMAN was slower and at least biphasic. About 85% of the enzyme were cleared with an apparent half time of 12 min, while the apparent clearance of the remaining fraction was about 47 min (Fig. 4).

The mice were killed 2 days after injection and extracts from liver, spleen, kidney and heart were examined for neutral oligosaccharides (Fig. 5). With the exception of liver the corrective effect was highest for the human LAMAN and lowest for the bovine LAMAN. The corrective effect of the mouse enzyme was intermediate. Only in liver was the corrective effect of the bovine enzyme (4% of the storage remaining) more pronounced than that of the human enzyme (14% of the storage remaining). In liver the corrective effect of mouse LAMAN was weakest (23% of the storage remaining).

#### Corrective effect of human LAMAN at high dose

The comparison of the bovine, mouse and human LAMAN indicated that the poorly phosphorylated human LAMAN had a relatively higher corrective potential in kidney and heart, two organs which are more resistant to metabolic correction than liver and spleen. To evaluate the corrective potential of the human enzyme we injected a single dose of 250 mU LAMAN and analysed the mice 1 to 12 days after injection. In liver storage was fully corrected 1 and 3 days after injection. After 6 and 12 days neutral oligosaccharides started

WO 2005/094874

PCT/DK2005/000228

45

to accumulate again, but reached only about 30% of the storage level before treatment (Fig. 6). Light microscopical examination of the liver revealed the almost complete disappearance of storage vacuoles, which are prominent in sinus endothelial cells, Kupffer cells and hepatocytes of untreated  $\alpha$ -mannosidosis mice and reappear 12 days after injection (Fig. 7). In spleen and kidney, the storage of neutral oligosaccharides decreased to 12 and 18%, respectively. It is noteworthy, that in spleen and kidney the maximum correction was observed after 3 and 6 days, respectively. In both organs neutral oligosaccharides started to reaccumulate 3 and 6 days after the injection (see Fig. 5). The neutral oligosaccharides in the brain of  $\alpha$ -mannosidosis mice were not affected by the treatment (not shown).

The human LAMAN activities recovered 24 h after injection were much higher than expected from the experiments with mouse LAMAN. In liver the human LAMAN activity was still 6 times higher than in control liver. In spleen and kidney it accounted for 10-15% of that in control. To follow the uptake and stability of human enzyme, LAMAN activity was determined in tissue extracts prepared 4, 16 and 24 h after injection of 250 mU human LAMAN per g body weight. Less than 20% of the injected LAMAN was recovered after 4 h in the tissues examined (liver 18%, kidney 0.4%, spleen 0.12% and heart 0.04%). When compared to the uptake of mouse LAMAN and taking into account that a 2.5 fold higher amount of human LAMAN was injected, the activity recovered in liver, kidney and heart 4 h after injection was comparable for both enzyme preparations (compare Table 1 and Table 2). Uptake by spleen appeared to be 2-3 times less efficient for the human enzyme. The biggest difference between the human and mouse enzyme was the higher stability of the human LAMAN internalized by liver, kidney and spleen. While the activity of the human LAMAN had decreased in these organs after 24 h to 17-33% of the 4 h value (see Table 2), that of the mouse enzyme had decreased to 20-26% of the 4 h value already after 10 h (see Table 1).

The corrective effect of the mouse (see Fig. 3) and human LAMAN was only transient. Neutral oligosaccharides started to reaccumulate 3 to 6 days after injection. Increasing the amount of injected enzyme would be a means to delay the reaccumulation. The corrective effect of a given dose of LAMAN is expected to be higher when administered as two half doses separated by an appropriate interval than as a single dose. Rather than increasing the amount of LAMAN to 500 mU/g body weight we administered two times 250 mU human LAMAN per g body weight each at day 7 and 3.5 prior to the analysis. This resulted in a full correction of the storage in kidney and heart (Fig. 8). Light microscopical examination demonstrated the absence of storage vacuoles in liver (not shown) and in tubular epithelia of kidney (Fig. 9). The residual storage in spleen was less than 20% of that in mock-injected  $\alpha$ -mannosidosis mice. Most notably, in brain the level of neutral

WO 2005/094874

PCT/DK2005/000228

46

oligosaccharides as quantified by TLC was only half of that in the brain of mock-injected  $\alpha$ -mannosidosis mice (Fig. 8).

| Hours after injection | LAMAN (mU/g wet weight)* |               |               |              |              |
|-----------------------|--------------------------|---------------|---------------|--------------|--------------|
|                       | <i>Liver</i>             | <i>Spleen</i> | <i>Kidney</i> | <i>Heart</i> | <i>Brain</i> |
| 4                     | 2774                     | 157           | 81            | 21           | 2,1          |
| 8                     | 2639                     | 65            | 45            | 8            | 1,7          |
| 16                    | 1549                     | 31            | 26            | 10           | 2,7          |
| 24                    | 796                      | 24            | 16            | 6            | 3,0          |

5 **TABLE 2 :** LAMAN activity in tissue extracts of  $\alpha$ -mannosidosis mice 4-24 h after injection of 250 mU of human  $\alpha$ -mannosidosis per g body weight.<sup>†</sup> All values were corrected for the mean LAMAN activity in serum at  $t_{\text{zero}}$  (5012 mU/ml serum). The correction factors were varied between 0,903 and 1,222.\* For LAMAN activity in tissue extracts of control mice and non-injected mice see Table1.

10

To quantify the neutral oligosaccharides on a molar basis the neutral oligosaccharides extracted from the brain of the mock- and LAMAN-injected  $\alpha$ -mannosidosis mice were reacted with 2-anthranilamide, which introduces a fluorescent tag at the reducing termini of the oligosaccharides. The fluorescently labelled oligosaccharides were separated by

15 HPLC and quantified by comparison with an internal standard oligosaccharide added prior to the derivatization with 2-anthranilamide (Fig. 10). The major oligosaccharide species in brain of  $\alpha$ -mannosidosis mice contain 2, 3 or 4 mannose residues. They are present at concentrations of 319, 87 and 164 pmol/g tissue, respectively, and account for 61% of all neutral oligosaccharides in brain. In the brain of treated  $\alpha$ -mannosidosis mice the

20 concentration of the neutral oligosaccharides decreased to 26% of mock-injected mice. The corrective effect was noted for each of the oligosaccharide species but was relatively highest for the species with 9, 7 and 4 mannose residues (see Fig. 10).

In the present study LAMAN purified from bovine kidney was used. This enzyme preparation consisted of mature polypeptides with a low mannose 6-phosphate content.

25 The recombinant human LAMAN isolated from the secretion of CHO cells consisted mainly of the precursor form, but was also poorly phosphorylated. The low mannose 6-phosphate



WO 2005/094874

PCT/DK2005/000228

47

content of this preparation is likely to be due to dephosphorylation during isolation. Occasionally, human LAMAN preparations were obtained of which up to 40% bound to the MRP 300 column (C. Andersson, D. P. Roces, unpublished observation). The recombinant mouse LAMAN purified from the secretions of mouse embryonic fibroblasts that lack

5 mannose 6-phosphate receptors, was largely phosphorylated and represented by the precursor form. The clearance from circulation was faster for the two poorly phosphorylated LAMAN preparations from bovine kidney and CHO cells than for the highly phosphorylated mouse LAMAN. The uptake of the different LAMAN preparations by organs was not directly compared. However, comparing the data of different experiments

10 suggests that of the poorly phosphorylated human and of the highly phosphorylated mouse LAMAN similar fractions are taken up by the liver, kidney and heart. Only in spleen was the uptake different and facilitated by higher phosphorylation. In a related study on the uptake of phosphorylated and non-phosphorylated  $\beta$ -glucuronidase in mice no difference was observed for the uptake into liver and spleen, while uptake into kidney and heart was

15 higher for the phosphorylated enzyme (26). For  $\alpha$ -galactosidase preparations that differed in glycosylation and phosphorylation a similar uptake by liver, spleen and kidney was observed (27). This indicates that the effect of glycosylation and phosphorylation on the uptake of lysosomal enzymes into different organs depends on the enzyme.

A major difference between the LAMAN preparations from different sources are their

20 stability. Assuming first order kinetics the activity of mouse (see Table 1) and bovine (not shown) LAMAN decreased in liver, kidney and spleen with an apparent half life of 3 h or less, while the apparent half life of human LAMAN was more than 3 times longer. This may explain the higher corrective efficacy of human LAMAN on the oligosaccharide storage in spleen, kidney and heart (see Fig. 5).

25 A single intravenous administration of LAMAN led to a rapid and pronounced decrease of the lysosomal storage of neutral oligosaccharides irrespective of the source of the enzyme. The corrective effect was most pronounced in liver. This is also the tissue where the increase of LAMAN activity relative to that in wild type mice was highest. Histological examination revealed a correction of storage in both parenchymal and non-parenchymal

30 liver cells. This was true for the highly phosphorylated mouse LAMAN (not shown) and for the poorly phosphorylated human LAMAN (see Fig. 7). This was unexpected as an earlier study with  $\beta$ -glucuronidase had shown that non-phosphorylated enzyme forms localized almost exclusively to non-parenchymal liver cells, while the phosphorylated forms localized to both parenchymal and non-parenchymal liver cells (26). The corrective effect of LAMAN

35 was transient in all tissues. The neutral oligosaccharides started to reaccumulate 2 to 6 days after the injection.

A remarkable correction of storage was observed when the injection of 250 mU human LAMAN per g body weight, which reduces storage in spleen and kidney by 80-90% was

WO 2005/094874

PCT/DK2005/000228

48

repeated after 3.5 days. Within one week the storage disappeared not only in liver, but also in kidney and heart. More importantly, the concentration of neutral oligosaccharides also decreased in brain to about one fourth. The latter cannot be attributed to an uptake of LAMAN into neural cells. In mice the blood-brain barrier matures within the first two weeks of life, and lysosomal enzymes administered intravenously after this period do not cross the blood-brain barrier (28-31). In accordance with these observations we observed trace amounts of LAMAN in the brain homogenates of treated mice (see Table 1 and 2). These activities are attributed to extraneural cells, such as endothelia, choroid plexus epithelium and incompletely removed meninges. As it is unlikely that the administered LAMAN gets access to oligosaccharides stored in neuronal and glial cells alternative mechanisms have to be considered. Clearance of oligosaccharides via the blood circulation or via an improved flow of the cerebrospinal fluid may contribute to the decrease of neutral oligosaccharide storage in brain.

#### Example 3: rhLAMAN - Efficacy of repeated dose replacement therapy in $\alpha$ -mannosidosis mice

The study objective is to

- Demonstrate a total correction of the mannose oligosaccharides storage in all the peripheral tissues, and verify the correction of the storage in the brain.
- Determine if the injection of a repeated dose of rhLAMAN has also an effect on the LAMAN activities measured in the different peripheral tissues.
- Follow the development of antibodies after the repeated injections, and check whether they precipitate the enzyme. Determine if, once the mice developed antibodies, these have an effect on the clearance of the enzyme.
- Determine whether an increased level of antibodies has an effect on the permeability of the brain blood barrier (BBB).
- Compare the gene expression of knock-out, injected knock-out and wild type mice after 1 and 4 weeks of treatment. A microarray experiment will be performed with RNA from liver. The mice used for this experiment are selected from heterozygote breedings (see below).

Justification of test system

Test item

The test item is supplied as an 5.4 mg.ml<sup>-1</sup> solution (8.2 U/mg, batch 041202) and is used as supplied. The test item is stored frozen (ca -20°C) in the dispensary when not in use.

WO 2005/094874

PCT/DK2005/000228

49

**Animals and management**

For the experiment 5 different groups of mice are used:

- (-/-)  $\alpha$ -mannosidosis mice
- (+/+) C57/B6 (wild type mice)
- 5 (+/-) heterozygote mice that are offspring of a cross between  $\alpha$ -mannosidosis mice (-/-) and C57/B6 mice(+/+)
- (-/-)  $\alpha$ -mannosidosis mice from heterozygote crosses (+/- mice)
- (+/+) wild type mice from heterozygote crosses (+/- mice)
- 10 Age: 8 weeks at initiation of the study.
- Number of Animals: 64 (males and/or females) mice
- Health Status: Animals suspected of being diseased are culled from the study. If significant numbers of animals are unsuitable, the entire batch of animals is rejected and a new batch obtained.
- 15 Acclimatisation: The animals are allowed to acclimatize to the accommodation for a minimum of 3 days prior to the commencement of dosing.

- Allocation to Dose Groups: All the animals are weighed and the required number of animals is randomly assigned to the 5 dose groups. A weight and/or sex stratification is not necessary, as the dose is weight adjusted, and no sex-specific differences were found
- 20 so far. Only males are used for the Microarray experiment (groups 2\*, 4\* and 5\*, see below), since in gene expression studies, sex has frequently been described as a relevant factor.

- Room Environment: The animals are housed 4/cage or 8/cage in a polypropylene cage with stainless steel mesh tops and solid bottoms with wood shavings as bedding material.
- 25 Cages (267 x 207 x 104 mm or 425 x 266 x 188 mm) are changed as necessary.

- The cages are supplied with a polycarbonate water bottle (capacity 250 ml) with a stainless steel nozzle and Durethane cap. Water bottles are filled as necessary and changed/washed at least once weekly.

30

There is automatic control of temperature and humidity, target ranges are 20°C $\pm$  2°C and 50% $\pm$ 15%, respectively, with a room airflow intended to give a minimum of 15 air changes per hour. Temperature and humidity ranges are monitored and recorded daily. There are automatic control of light cycle; light hours are normally 0600-1800.

WO 2005/094874

PCT/DK2005/000228

50

### Diet and Water Supply

Food and water are available to the animals *ad libitum*. The food and water used are not considered to contain any additional substances, in sufficient concentration, to have any influence on the outcome of the study.

## 5 Treatment

### Dose Groups/Dose Level

Group 1 (for biochemistry investigations, 16 LAMAN mice). Mice are treated twice a week intravenously with 250 mU/g body weight rhLAMAN, and groups of 4 mice are killed after  
10 1, 2 and 4 weeks of treatment.

Group 2\* (for microarray investigations, 8 LAMAN mice from heterozygous cross), are treated twice a week intravenously with 250 mU/g body weight rhLAMAN, and groups of 3 mice are killed after 1 and 4 weeks of treatment.

Group 3 (for blood-brain-barrier, Antibody and plasma clearance investigations, 12 LAMAN  
15 mice), are treated once weekly with 250 mU/g body weight rhLAMAN. Both groups of mice, the 4 mice used for BBB Investigations, and the 4 mice used for clearance investigation, are killed after 4 weeks of treatment.

Group 4 (Mock treated mice, 12 LAMAN mice) are treated once/twice weekly with vehicle for rhLAMAN at the same dose volume as the treated animals in Group 3/Group 1. A group  
20 of 4 mice once a week injected are killed after 4 weeks of treatment (to be compared with mice from group 3), and groups of 2 mice are killed after 1, 2 and 4 weeks of treatment (to be compared with group 1)

Group 4\* (Mock treated mice, 8 LAMAN mice from heterozygous cross) and Group 5\* (8  
wild type from heterozygous cross) are treated twice a week with vehicle for rhLAMAN at  
25 the same dose volume as the treated animals in Group 2\*. Groups of 3 mice are killed after 1 and 4 weeks.

\* $\alpha$ -Mannosidosis mice from heterozygote breedings (+/-), offspring of a breeding between  $\alpha$ -mannosidosis mice (-/-) and wild type C57/B6 (+/+).

WO 2005/094874

PCT/DK2005/000228

51

TABLE 3: Allocation of animals to dose groups:

| Animals                |                  | LAMAN knock-out mice<br>(LAMAN injected) |   |    |            |     |               |             | No<br>of<br>Injec-<br>tions |
|------------------------|------------------|------------------------------------------|---|----|------------|-----|---------------|-------------|-----------------------------|
| Group No               |                  | 1A                                       |   | 1C | 2A*        | 2B* | 3A            | 3B          |                             |
| Treat-<br>ment<br>time | Killing<br>times |                                          |   |    | Microarray |     | Evans<br>Blue | Ab-<br>mice |                             |
| t <sub>0</sub>         |                  | 36x                                      | + | +  | +          | +   | +             | +           | 36x                         |
| t <sub>0,5w</sub>      |                  | 24x                                      | + | +  | +          | +   |               |             | 24x                         |
| t <sub>1w</sub>        | t <sub>1w</sub>  | 29x                                      | + | +  | 3*         | +   | +             | +           | 29x                         |
| t <sub>1,5w</sub>      |                  | 17x                                      | + | +  |            | +   |               |             | 17x                         |
| t <sub>2w</sub>        | t <sub>2w</sub>  | 25x                                      | 4 | +  |            | +   | +             | +           | 25x                         |
| t <sub>2,5w</sub>      |                  | 13x                                      |   | +  |            | +   |               |             | 13x                         |
| t <sub>3w</sub>        |                  | 25x                                      |   | +  |            | +   | +             | +           | 25x                         |
| t <sub>3,5w</sub>      |                  | 13x                                      |   | +  |            | +   |               |             | 13x                         |
| t <sub>4w</sub>        | t <sub>4w</sub>  | -                                        |   | 4  |            | 3*  | 4             | 4           | -                           |
|                        |                  |                                          |   |    |            |     |               |             |                             |
| No                     |                  |                                          | 4 | 4  | 3          | 3   | 4             | 4           |                             |
| Spar<br>e              |                  |                                          |   | +4 |            | +2  |               | +4          |                             |

5

TABLE 3: Allocation of animals to dose groups (continued)

| Animals                |                  | LAMAN knock-out mice    |    |    |    |     |     | Wild Type<br>mice    | No<br>of<br>Injec-<br>tions |
|------------------------|------------------|-------------------------|----|----|----|-----|-----|----------------------|-----------------------------|
| Group No               |                  | 4A                      | 4B | 4C | 4D | 4E* | 4F* | 5A*                  | 5B*                         |
| Treat-<br>ment<br>time | Killing<br>times | Control<br>Mock treated |    |    |    |     |     | Controls<br>Mock Tr. |                             |
| t <sub>0</sub>         |                  | +                       | +  | +  | +  | +   | +   | +                    | +                           |
| t <sub>0,5w</sub>      |                  | +                       | +  | +  |    | +   | +   | +                    | +                           |

WO 2005/094874

PCT/DK2005/000228

52

|                   |                 |   |   |   |   |    |    |    |    |     |
|-------------------|-----------------|---|---|---|---|----|----|----|----|-----|
| t <sub>1w</sub>   | t <sub>1w</sub> | 2 | + | + | + | 3* | +  | 3* | +  | 29x |
| t <sub>1,5w</sub> |                 |   | + | + |   |    | +  |    | +  | 17x |
| t <sub>2w</sub>   | t <sub>2w</sub> |   | 2 | + | + |    | +  |    | +  | 25x |
| t <sub>2,5w</sub> |                 |   |   | + |   |    | +  |    | +  | 13x |
| t <sub>3w</sub>   |                 |   |   | + | + |    | +  |    | +  | 25x |
| t <sub>3,5w</sub> |                 |   |   | + |   |    | +  |    | +  | 13x |
| t <sub>4w</sub>   | t <sub>4w</sub> |   |   | 2 | 4 |    | 3* |    | 3* | -   |
|                   |                 |   |   |   |   |    |    |    |    |     |
| No                |                 | 2 | 2 | 4 | 2 | 3  | 3  | 3  | 3  |     |
| Spare             |                 |   |   |   |   |    | +4 |    | +2 |     |

#### Route, Duration and Method of Administration

The test item is administered via an intravenous (bolus) injection to one of the lateral tail veins at target dose levels. The requisite dose volume is calculated according to the

5 animals most recently recorded body weight.

Prior to the administration, the mice are anesthetized (intra peritoneal) with 75 µl per 100 g body weight of a solution 10 mg/ml Ketavet (Parke Davis) and 2 mg/ml Rompun (Bayer), and are kept by 37 °C until they are recovered from anesthesia.

#### Observations

##### 10 Viability

All animals are checked early morning and as late as possible each day for viability. Any animal showing signs of severe debility or intoxication and considered to be in a moribund state, is killed. Any animal killed in extremis or found dead are, whenever possible, subjected to a macroscopic post mortem examination on the day of death. If it is not  
15 feasible to undertake the macroscopic post mortem examination on the day of death (eg late afternoon or weekends), the carcass is placed in a polythene bag and kept in a refrigerator (4°C) until examination the following day.

#### Clinical Signs

All animals are observed on days of dosing for reaction to treatment. The onset, intensity  
20 and duration of any signs observed is recorded.

#### Body Weight

Prior to dosing all animals are weighted weekly and recorded and tabulated.

WO 2005/094874

PCT/DK2005/000228

53

## Laboratory Investigations

### Treatment verification

Five minutes after injection, blood from the retroorbital plexus is taken for measurement of rhLAMAN plasma levels (according to internal procedure no 2). If an animal did not receive  
5 the full dose, the % missing amount is calculated and given to the animal on the same day.

### Kinetics

From 4 mice of group 3, blood from the retroorbital plexus is collected one day before the injection, and also 5, 15, and 30 minutes after each injection, to investigate a possible  
10 effect of the Ab on the clearance of the enzyme.

When enzyme is still available at the end of the experiment (4 weeks), the treatment of these mice will continue up to 8 weeks.

### Antibody determination

Before dosing blood from retroorbital plexus is taken from group 2 animals as well as of all  
15 other animals at termination in order to measure the antibody titers.

### Terminal studies

#### Method of killing

Mice are anaesthetized with 600 µl of a solution 10 mg/ml Ketavet (Parke Davis) and 2 mg/ml Rompun (Bayer) in 0.15M NaCl, perfused with PBS and killed by bleeding.

20 For all groups except group 3, the killing take place three and a half days after last treatment. For group 3, the mice are killed 1 week after last treatment (see table 3).

#### Histopathology

The following organs are collected and fixed with 0.1mM NaPi/6% Gluteraldehyd buffered to pH 7.4: liver, kidney, brain

25

Small sections are cut with razor blade, and the fixed slides are subject to electron microscopy analysis.

#### Tissue LAMAN concentrations

Homogenates of Liver, Spleen, kidney, heart, brain in TBS/PI (Protease Inhibitors) are  
30 measured for LAMAN activity using p-nitrophenyl- $\alpha$ -mannopyranoside as substrate and 0,2M sodium citrate pH 4.6, 0,08% Na<sub>3</sub>, 0,4% BSA as buffer.

WO 2005/094874

PCT/DK2005/000228

54

Processing of LAMAN is followed by Western Blot (~1mU is needed).

#### Tissue Oligosaccharides

From the organs described in 10.3 a preparation of neutral oligosaccharides extracts are  
5 prepared and quantified by densitometry to study the normalization of the storage in the  
organs by TLC according to procedure no 4 and 5.

Neutral oligosaccharides from liver and heart will be digested with  $\alpha$ -glucosidase, to avoid  
the interference of glucogen.

#### Statistical analysis of results

- 10 Unless otherwise stated, all statistical tests will be two-sided and performed at the 5%  
significance level using in-house software.

The following pairwise comparisons are performed:

- 15 Vehicle Control Group 4A vs Group 1A  
Vehicle Control Group 4B vs Group 1B  
Vehicle Control Group 4C vs Group 1C  
Vehicle Control Group 4D vs Group 3A  
Vehicle Control Group 5A\*, 5B\* vs Group 4E\*, 4F\*  
20 Vehicle Control Group 5A\*, 5B\* vs Group 2A\*, 2B\*  
Vehicle Control Group 4E\*, 4F\* vs Group 2A\*, 2B\*

The data are analysed for normality of the distribution using the Shapiro-Wilk test and  
quantile-quantile-plots. When a normal distribution can be assumed, differences between  
25 the groups are tested for using linear models (ANOVA) with or without homoscedasticity  
assumption. If the data (or log transformed data) are not normally distributed, a Kruskal-  
Wallis-Test is used. Additional data analysis steps may be undertaken depending on the  
data.

(\* heterozygote breedings, see below)

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WO 2005/094874

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WO 2005/094874

PCT/DK2005/000228

61

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WO 2005/094874

PCT/DK2005/000228

62

## CLAIMS

1. Use of a lysosomal alpha-mannosidase for the preparation of a medicament for reducing the intracellular levels of neutral mannose-rich oligosaccharides in cells within one or more regions of the central nervous system, wherein said medicament does not comprise a component with a known ability to target cells within the central nervous system
2. Use according to claim 1, wherein the lysosomal alpha-mannosidase is selected from the group consisting of
  - (a) the amino acid sequence of SEQ ID NO:1;
  - 10 (b) a portion of the sequence in (a), which is enzymatically equivalent to recombinant human lysosomal alpha-mannosidase
  - (c) an amino acid sequence analogue having at least 75% sequence identity to any one of the sequences in (a) or (b) and at the same time being enzymatically equivalent to recombinant human lysosomal alpha-mannosidase.
- 15 3. Use according to claim 1 or 2, wherein the lysosomal alpha-mannosidase is formulated for systemic delivery.
4. Use according to any of the preceding claims, wherein the lysosomal alpha-mannosidase has not been modified in order to obtain carrier-mediated transport across the blood-brain barrier.
- 20 5. Use according to any of the preceding claims, wherein the lysosomal alpha-mannosidase has not been formulated so as to obtain disruption of the blood-brain barrier.
6. Use according to any of the preceding claims, wherein said medicament comprises a lysosomal alpha-mannosidase and does not comprise
  - a) a vehicle or vector, such as a peptide or polypeptide, for delivery of lysosomal
  - 25 alpha-mannosidase into the central nervous system, and
  - b) a component with a known ability to cause opening or disruption of the blood brain barrier, and
  - c) an intact cell
7. Use according to any of the preceding claims, wherein said medicament does not
- 30 comprise a vehicle selected from the group consisting of exogenous peptides and proteins, including the insulin fragment and peptides and proteins derived from virus or bacteria, polyamines, lipophilic moieties including phospholipids, antibodies or antibody fragments such as transferrin receptor antibodies and toxins.



WO 2005/094874

PCT/DK2005/000228

63

8. Use according to any of the preceding claims, wherein said medicament is formulated in an isotonic solution.
9. Use according to any of the preceding claims, wherein said medicament is formulated in a buffer comprising/consisting of 3.10-3.50 mM  $\text{Na}_2\text{HPO}_4$ , 0.4-0.6 mM  $\text{NaH}_2\text{PO}_4$ , 25-30 mM Glycine, 220-250 mM Mannitol, and water for injection.
10. Use according to any of the preceding claims, wherein said medicament is formulated in a buffer comprising/consisting of 4.0-5.0 mM Sodium Citrate, 0.3-0.8 mM Citric Acid, 200-250 mM Mannitol, 3.0-5.0 mM Tween 80, and water for injection.
- 10 11. Use according to any of the preceding claims, wherein said medicament does not comprise an intact cell selected from the group consisting of a transduced autologous cell, such as a transduced fibroblast or a peripheral blood lymphocyte and polymer encapsulated cell line capable of secreting alpha-mannosidase into the cerebrospinal fluid.
12. Use according to any of the preceding claims, wherein said medicament is for  
15 administration by intravenous or subcutaneous injection.
13. Use according to any of the preceding claims, wherein said medicament is for administration to a subject, which does not receive any additional treatment in order to reduce the levels of neutral oligosaccharides, including bone marrow transplantation, interstitial delivery of lysosomal alpha-mannosidase, treatment in order to obtain  
20 disruption of the blood-brain barrier and treatment in order to obtain carrier-mediated transport across the blood-brain barrier.
14. Use according to any of the preceding claims wherein said medicament is administered by repeated injection on a daily basis, by repeated injection every 2, 3, 4, 5, or 6 days, or by repeated injection on a weekly, bi-weekly or monthly basis.
- 25 15. Use according to any of the preceding claims, wherein the amount of lysosomal alpha-mannosidase administered is such that:
- a) effective levels of the enzyme are sustained in circulation for not less than 5 – 30 minutes such as not less than 5, 10, 15, 20, 25, or 30 minutes, and/or
- 30 b) effective levels of the enzyme are sustained in visceral organs, including the kidney, spleen and heart, for not less than 4 hours, and/or
- c) effective levels of the enzyme are sustained in the liver for not less than 4 days, subsequent to injection of said medicament.
- 35 16. Use according to any of the preceding claims, wherein the amount of lysosomal alpha-mannosidase administered is such that:

WO 2005/094874

PCT/DK2005/000228

64

- 5 a) the amount of neutral oligosaccharides in visceral organs including the liver, spleen kidney and heart is reduced by 25 – 95% over 10 hours, and/or
- b) the amount of neutral oligosaccharides in visceral organs including the liver, spleen kidney and heart is reduced by 55 – 95% over a period of 2 to 6 days , and/or
- c) the storage of neutral oligosaccharides in the liver is reduced over a period of 1 to 12 days as determined by a reduced presence of storage vacuoles within the sinus endothelial cells, Kupffer cells and hepatocytes subsequent to injection of said medicament.

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17. Use according to any of the preceding claims, wherein the amount of lysosomal alpha-mannosidase administered is such that:

- 15 a) effective levels of the enzyme are sustained in circulation, and/or
- b) effective levels of the enzyme are sustained in visceral organs, including the kidney, spleen and heart, and/or
- c) effective levels of the enzyme are sustained in the liver,
- by repeated injection of the medicament on a daily basis, by repeated injection every 2, 3, 4, 5, or 6 days, or by repeated injection on a weekly, bi-weekly or monthly basis.

20 18. Use according to any of the preceding claims, wherein the amount of lysosomal alpha-mannosidase administered is such that

- a) the storage of neutral oligosaccharides in kidney and heart is fully corrected, and/or
- b) the amount of neutral oligosaccharides in the spleen is reduced by at least 80%, and/or
- 25 c) the storage of neutral oligosaccharides in the liver and the kidney is reduced as determined by a reduced presence of storage vacuoles within the sinus endothelial cells, Kupffer cells and hepatocytes of the liver and in the tubular epithelia of the kidney
- by repeated injection of the medicament on a daily basis, by repeated injection every 2, 3, 4, 5, or 6 days, or by repeated injection on a weekly, bi-weekly or monthly basis.

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19. Use according to any of the preceding claims wherein said medicament is administered in an amount, which is within the range of 1 to 400 mU lysosomal alpha-mannosidase per gram body weight of the subject to be treated.

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20. Use according to any of the preceding claims, wherein said lysosomal alpha-mannosidase has a specific activity of 5 - 100 U/mg.

WO 2005/094874

PCT/DK2005/000228

65

21. Use according to any of the preceding claims, wherein at least 30% of the amount of lysosomal alpha-mannosidase is present in the form of a 260 kDa dimer composed of 130 kDa monomers of an amino acid sequence having at least 75% sequence identity to SEQ ID NO: 1
- 5 22. Use according to any of the preceding claims, wherein a fraction the amount of said lysosomal alpha-mannosidase consists of lysosomal alpha-mannosidase having one or more N-linked oligosaccharides carrying mannose 6-phosphate residues.
23. Use according to any of the preceding claims, wherein said lysosomal alpha-mannosidase has a content of mannose 6-phosphate residues allowing mannose 6-phosphate dependent binding of from 2 to 100% of the amount of enzyme to a Man6P-receptor matrix.
- 10 25. Use according to any of the preceding claims, wherein a fraction constituting from 2 – 100% of the amount of said lysosomal alpha-mannosidase binds to the Man-6-P-receptor with high affinity.
- 15 26. Use according to any of the preceding claims, wherein said lysosomal alpha-mannosidase is a recombinant enzyme.
27. Use according to any of the preceding claims, wherein said lysosomal alpha-mannosidase is produced in a mammalian cell system.
- 20 28. Use according to claim 27, wherein said mammalian cell system is selected from the group consisting of CHO, BHK and COS cells.
29. Use according to claim 27, wherein said mammalian cell system is a human fibroblast cell line.
- 25 30. A method of reducing the intracellular levels of neutral mannose-rich oligosaccharides in cells within one or more regions of the central nervous system of a subject, said method comprising administering to said subject a formulation of lysosomal alpha-mannosidase, which does not comprise a component with a known ability to target the central nervous system.
- 30 31. A method according to claim 30, said method comprising administering said formulation of alpha-mannosidase by a route other than intracerebroventricular, spinal, intrathecal or intracranial administration.
32. A method according to claim 30 or 31, wherein the formulation is administered by intravenous injection.

**WO 2005/094874****PCT/DK2005/000228**

66

33. A method according to any of claims 30 to 32, wherein the administration of said preparation of lysosomal alpha-mannosidase is not supplemented by the administration of a composition comprising a component with a known ability to target the central nervous system.

- 5 34. A formulation of a lysosomal alpha-mannosidase, which does not comprise a component with a known ability to target cells within the central nervous system, for use as a medicament for reducing the intracellular levels of neutral mannose-rich oligosaccharides in cells within one or more regions of the central nervous system.

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WO 2005/094874

PCT/DK2005/000228

1/10

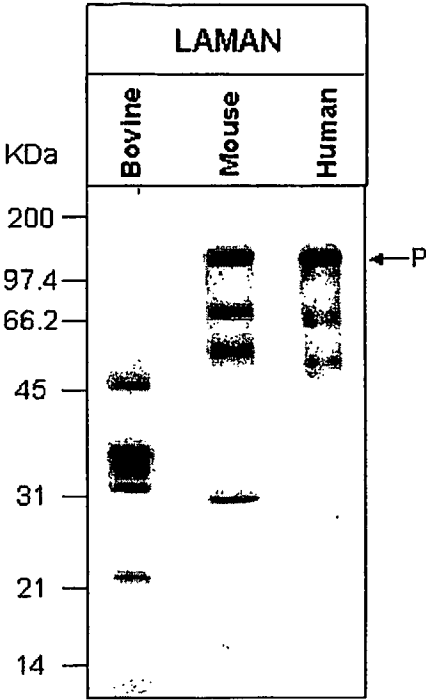


Fig. 1

WO 2005/094874

PCT/DK2005/000228

2/10

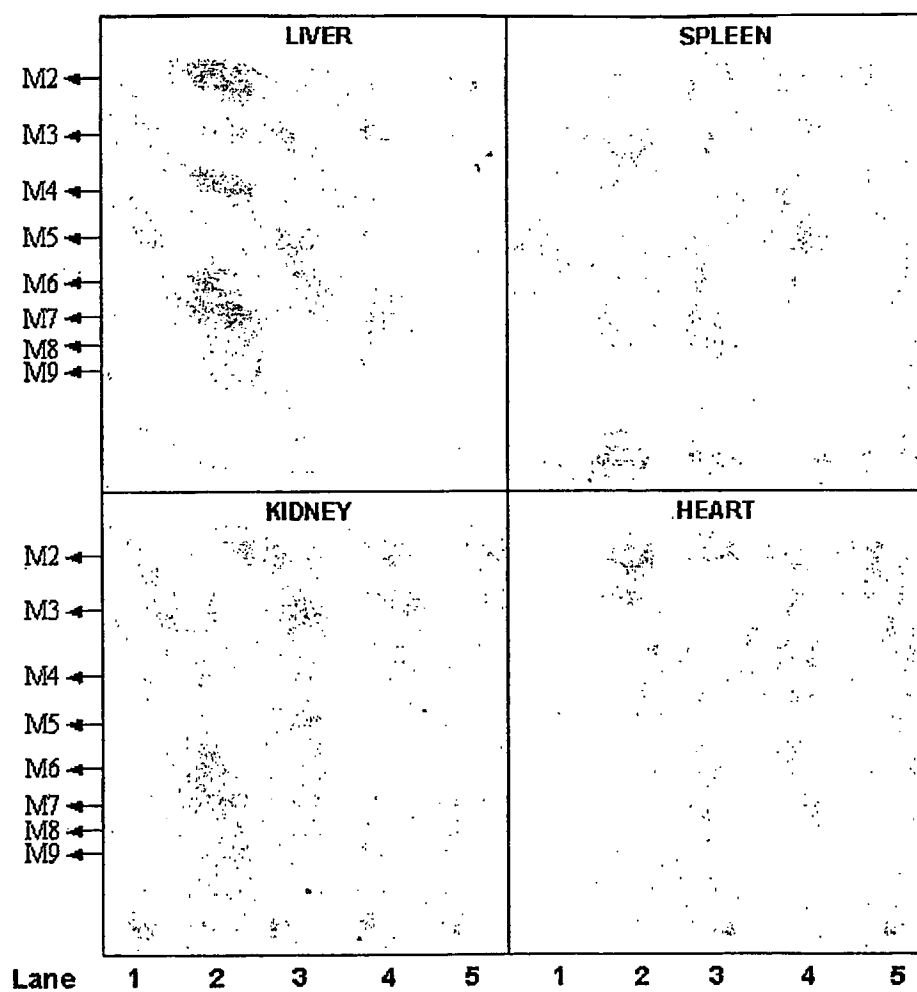


Fig. 2

WO 2005/094874

PCT/DK2005/000228

3/10

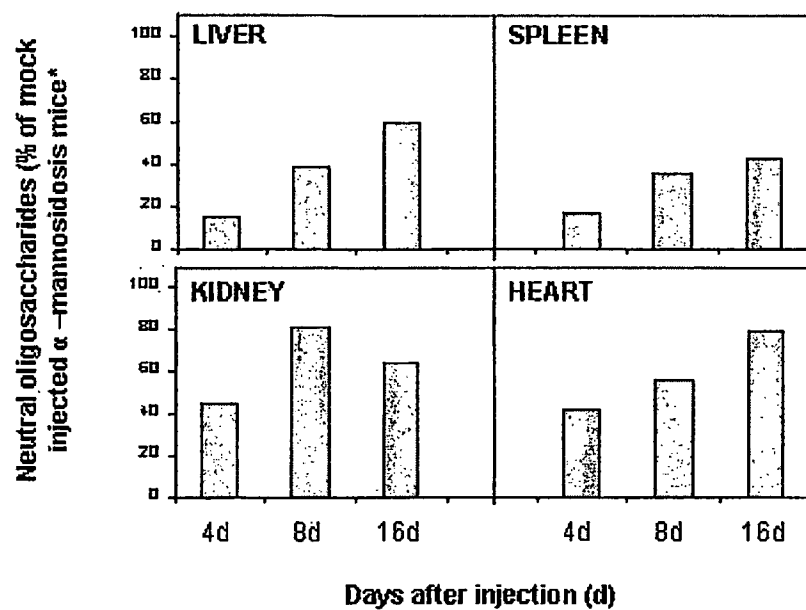


Fig. 3

WO 2005/094874

PCT/DK2005/000228

4/10

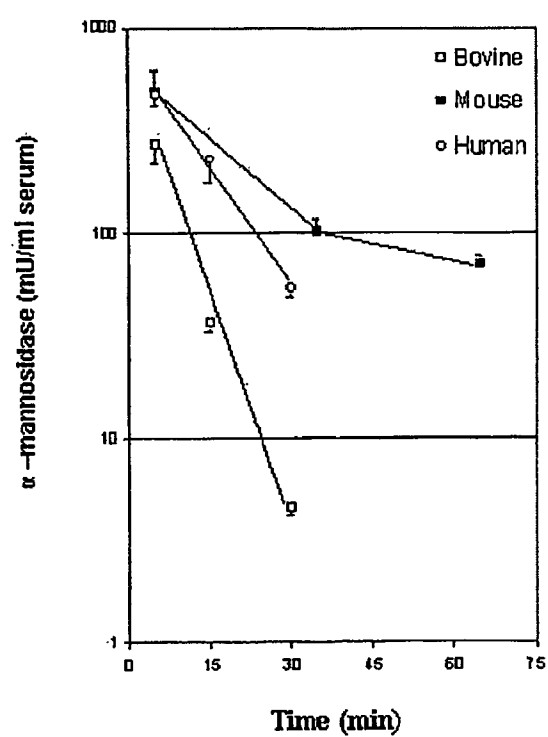


Fig. 4



WO 2005/094874

PCT/DK2005/000228

5/10

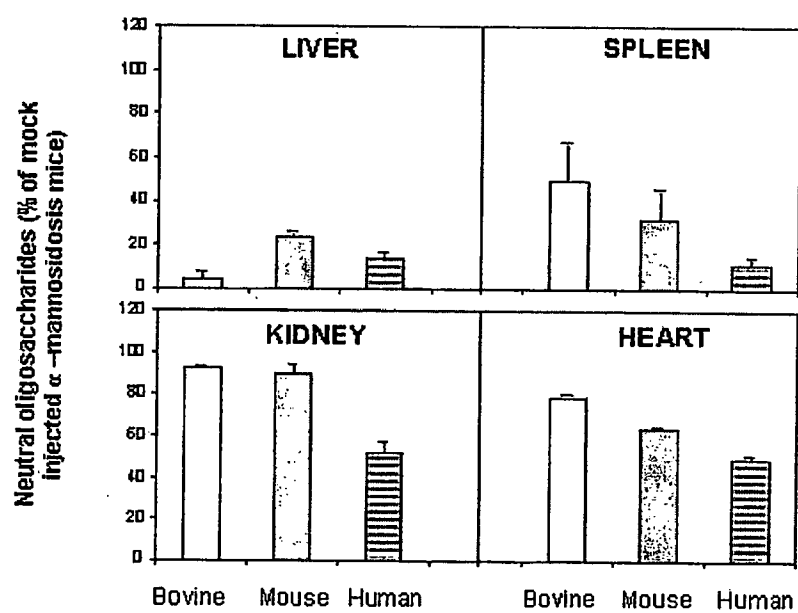


Fig. 5

6/10

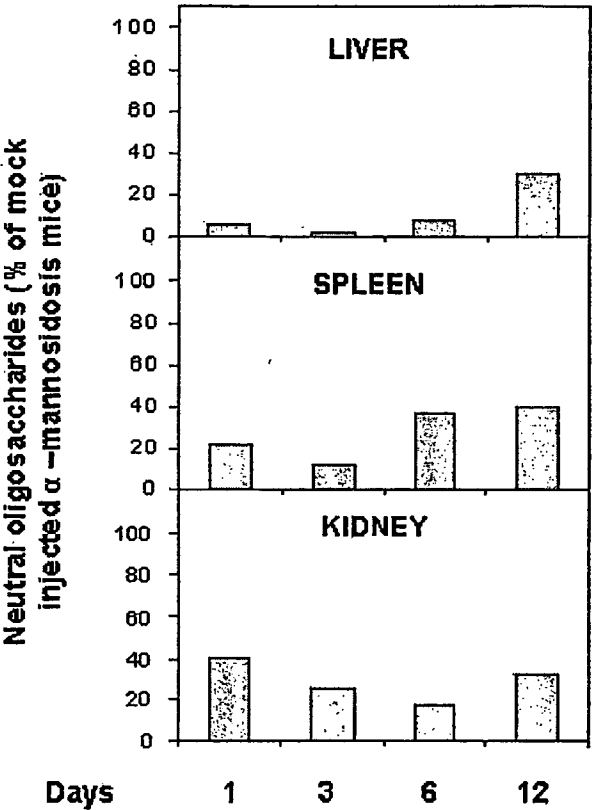


Fig. 6

WO 2005/094874

PCT/DK2005/000228

7/10

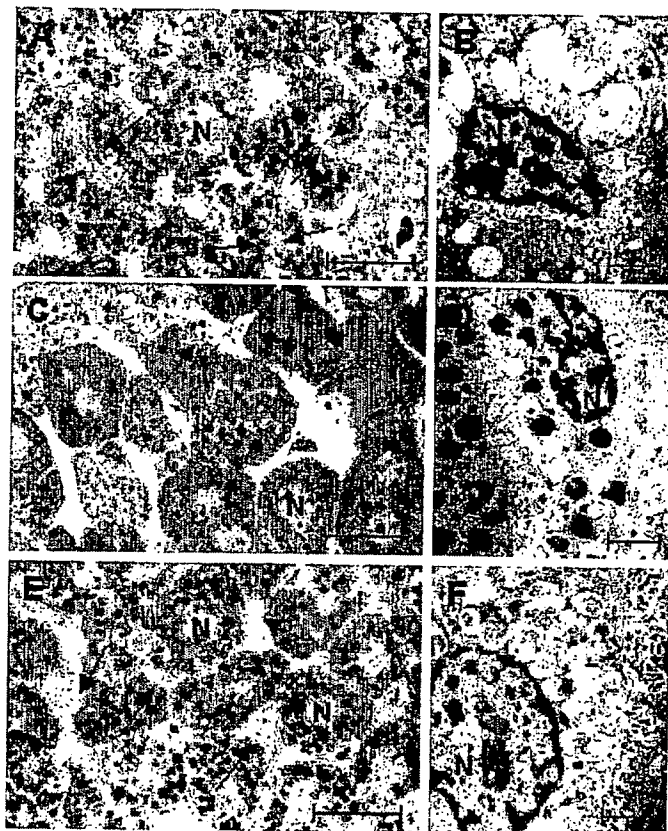


Fig. 7

WO 2005/094874

PCT/DK2005/000228

8/10

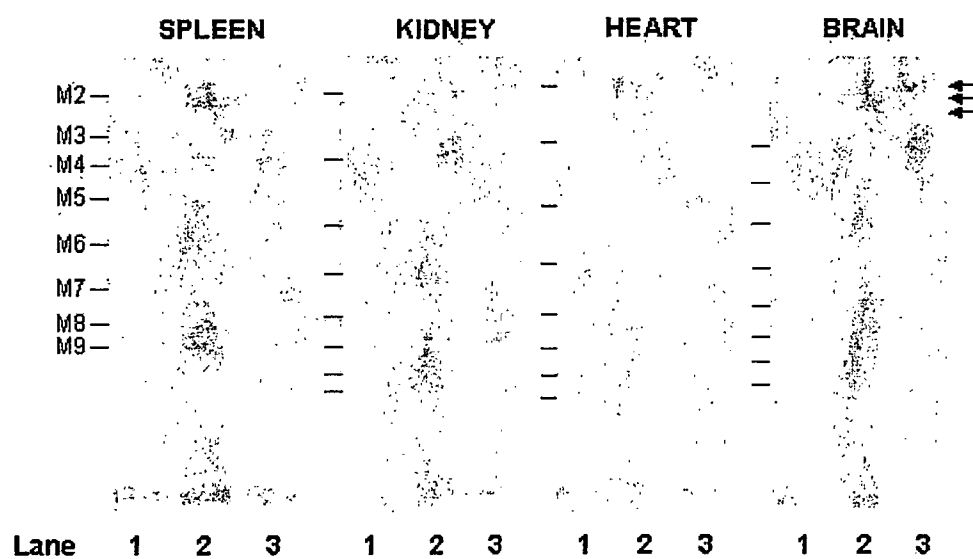


Fig. 8

WO 2005/094874

PCT/DK2005/000228

9/10

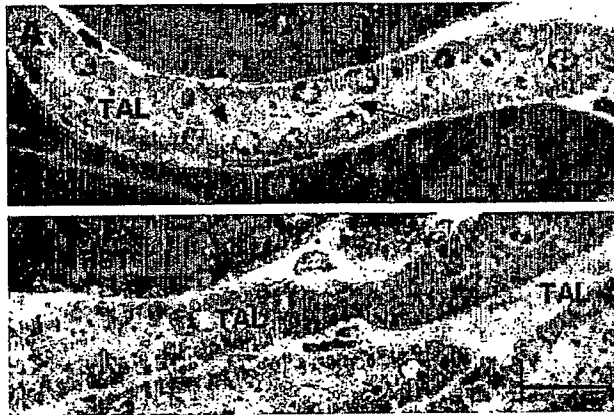


Fig. 9

WO 2005/094874

PCT/DK2005/000228

10/10

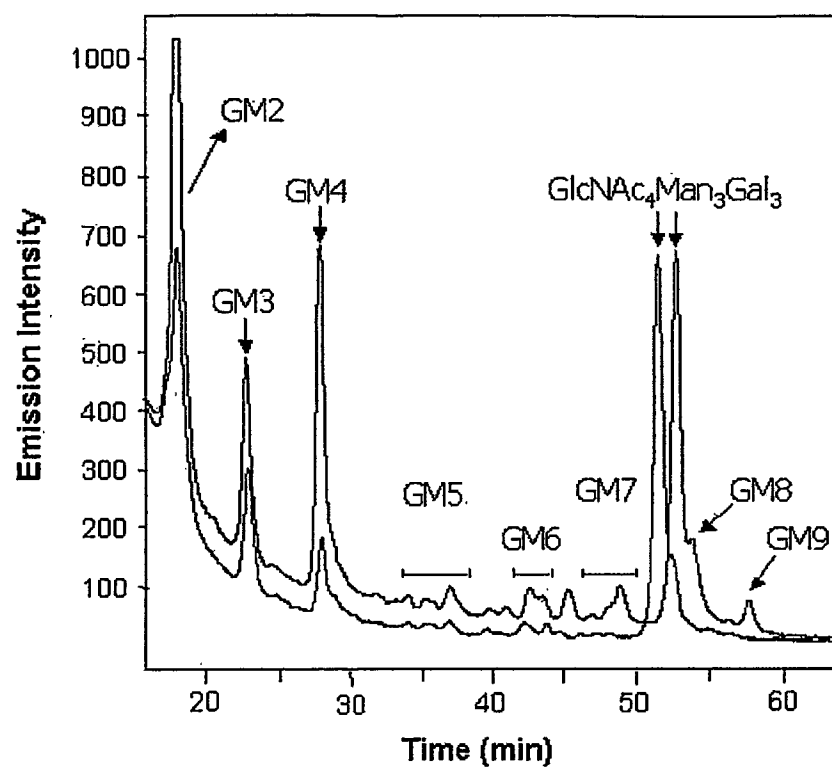


Fig. 10

WO 2005/094874

PCT/DK2005/000228

1/7

## SEQUENCE LISTING

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Claes Anderson  
Cecilia Weigelt  
Diego Prieto Roces  
Kurt von Figura  
Meher Irani

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correction of abnormal lysosomal storage in the CNS

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WO 2005/094874

PCT/DK2005/000228

2/7

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WO 2005/094874

PCT/DK2005/000228

3/7

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WO 2005/094874

PCT/DK2005/000228

4/7

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WO 2005/094874

PCT/DK2005/000228

5/7

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WO 2005/094874

6/7

PCT/DK2005/000228

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WO 2005/094874

PCT/DK2005/000228

7/7

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## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/DK2005/000228

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 A61K38/46 A61P25/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, Sequence Search

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                       | Relevant to claim No. |
|------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X          | <p>WO 02/099092 A (FOGH JENS ; HEMEBIOTECH AS (DK); ANDERSSON CLAES (SE); WEIGELT CECILIA) 12 December 2002 (2002-12-12)<br/>*SEQ ID NO: 12 (human LAMAN) is identical with SEQ ID NO:1 of the application*<br/>page 1, line 1 - line 22<br/>page 5, line 1 - line 14<br/>page 6, line 19 - line 37<br/>page 33, line 28 - page 34, line 4; claims</p> <p style="text-align: center;">-----<br/>-/--</p> | 1-34                  |



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the International filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the International filing date but later than the priority date claimed

"T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the International search

2 August 2005

Date of mailing of the International search report

08/08/2005

Name and mailing address of the ISA

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Authorized officer

Bayrak, S

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/DK2005/000228

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                                                                | Relevant to claim No. |
|------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X          | <p>BERG THOMAS ET AL: "Purification and characterization of recombinant human lysosomal alpha-mannosidase"<br/>MOLECULAR GENETICS AND METABOLISM,<br/>ACADEMIC PRESS, SAN DIEGO, CA, US,<br/>vol. 73, no. 1,<br/>24 April 2001 (2001-04-24), pages 18-29,<br/>XP002182076<br/>ISSN: 1096-7192<br/>page 25, column 1, paragraph 2 - page 25,<br/>column 2, paragraph 2<br/>page 27, column 1, paragraph 2 - column 2,<br/>paragraph 1; table 2</p> | 1-34                  |
| Y          | <p>BERG A T ET AL: "alpha-Mannosidosis in the guinea pig: cloning of the lysosomal alpha-mannosidase cDNA and identification of a missense mutation causing alpha-mannosidosis"<br/>BIOCHIMICA ET BIOPHYSICA ACTA. MOLECULAR BASIS OF DISEASE, AMSTERDAM, NL,<br/>vol. 1586, no. 2,<br/>16 March 2002 (2002-03-16), pages 169-176,<br/>XP004349023<br/>ISSN: 0925-4439<br/>page 175, column 1, paragraph 3 - column 2, paragraph 1</p>            | 1-34                  |
| Y          | <p>MERKLE R K ET AL: "Cloning, expression, purification, and characterization of the murine lysosomal acid alpha-mannosidase"<br/>BBA - GENERAL SUBJECTS, ELSEVIER SCIENCE PUBLISHERS, NL,<br/>vol. 1336, no. 2,<br/>27 August 1997 (1997-08-27), pages 132-146, XP004276600<br/>ISSN: 0304-4165<br/>page 145, column 1, paragraph 1 - paragraph 2</p>                                                                                            | 1-34                  |
| Y          | <p>GONZALEZ D S ET AL: "Isolation and characterization of the gene encoding the mouse broad specificity lysosomal alpha-mannosidase"<br/>BIOCHIMICA ET BIOPHYSICA ACTA . GENE STRUCTURE AND EXPRESSION, ELSEVIER, AMSTERDAM, NL,<br/>vol. 1445, no. 1,<br/>14 April 1999 (1999-04-14), pages 177-183,<br/>XP004275375<br/>ISSN: 0167-4781<br/>page 183, column 1, paragraph 2</p>                                                                 | 1-34                  |

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/DK2005/000228

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages                                                                       | Relevant to claim No. |
|------------|----------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A          | WO 00/73482 A (UNIV IOWA RES FOUND ;<br>CHIRON CORP (US); GHODSI ADBI (US);<br>DERKSEN TODD) 7 December 2000 (2000-12-07)<br>the whole document<br>----- | 1-34                  |



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/DK2005/000228**Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
**Although claims 30-33 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.**
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No  
PCT/DK2005/000228

| Patent document<br>cited in search report |   | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|-------------------------------------------|---|---------------------|----------------------------|---------------------|
| WO 02099092                               | A | 12-12-2002          | WO 02099092 A2             | 12-12-2002          |
|                                           |   |                     | US 2003199073 A1           | 23-10-2003          |
| WO 0073482                                | A | 07-12-2000          | AU 5295500 A               | 18-12-2000          |
|                                           |   |                     | CA 2375123 A1              | 07-12-2000          |
|                                           |   |                     | EP 1183384 A1              | 06-03-2002          |
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|                                           |   |                     | WO 0073482 A1              | 07-12-2000          |
|                                           |   |                     | US 2003223963 A1           | 04-12-2003          |
|                                           |   |                     | US 6730297 B1              | 04-05-2004          |